International Doctoral Thesis / Tesis Doctoral Internacional

The microbiome of the male reproductive tract: uncovering its composition and origins

El microbioma del tracto reproductor masculino:
desvelando su composición y orígenes

UNIVERSIDAD DE GRANADA

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The microbiome of the male reproductive tract: uncovering its composition and origins

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2023

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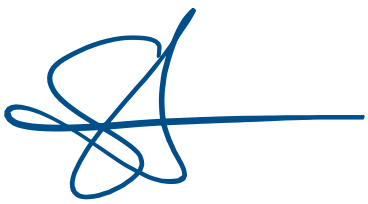

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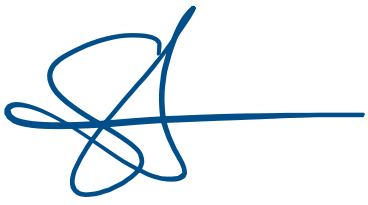

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La doctoranda Dª. Nerea Molina Morales ha realizado la presente Tesis Doctoral Internacional siendo beneficiaria de un contrato predoctoral del programa de Formación de Profesorado Universitario (referencia: FPU19/01638) del Ministerio de Universidades, por Resolución de 16 de octubre de 2019 de la Secretaría de Estado de Universidades, Investigación, Desarrollo e Innovación, en el Departamento de Bioquímica y Biología Molecular I de la Universidad de Granada.

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A las niñas y el niño

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LIST OF ABBREVIATIONS

AIDS: acquired immunodeficiency deficiency syndrome

ANCOM-BC: Analysis of Compositions of Microbiomes with Bias Correction

ANOVA: analysis of variance

ART: assisted reproductive technology

ASV: amplicon sequence variant

Avg: average

BMI: body mass index

BV: bacterial vaginosis

CE: chronic endometritis

CFU colony-forming units

COS: controlled ovarian stimulation

DADA2: Divisive Amplicon Denoising Algorithm 2

ENA: European Nucleotide Archive

EP: endometrial polyps

ET: embryo transfer

FDR: false discovery rate

FSH: follicular-stimulating hormone

GU: gonococcal

HIV: human immunodeficiency virus

HMP: Human Microbiome Project

HPV: human papillomavirus

HSV: herpes simplex virus

iHMP: integrative Human Microbiome Project

ITS: internal transcribed spacer

IUI: intrauterine insemination

IVF: in vitro fertilisation

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MeSH: medical subject heading

Meta-RNAseq: metatranscriptome analysis

MIMARKS: minimum information about a marker gene sequence

MIMS: minimum information about a metagenome sequence

mRNA: messenger RNA

MSM: men who have sex with men

NA: not applicable

NCBI: National Center for Biotechnology Information

NGS: next-generation sequencing

NGU: non-gonococcal

NIH: National Institutes of Health

NMDS: nonmetric multidimensional scaling

NR: non reported

OTU: operational taxonomic unit

PCR: polymerase chain reaction

PERMANOVA: permutational analysis of variance

Phy: phylotype

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PROSPERO: International Prospective Register of Systematic Reviews

QIIME2: Quantitative Insights Into Microbial Ecology 2

qPCR: quantitative polymerase chain reaction

RCT: randomised controlled trials

RDP: Ribosomal Database Project

RIF: recurrent implantation failure

RPL: recurrent pregnancy loss

rRNA: ribosomal RNA

SD: standard deviation

spp.: species

SRA: Sequence Read Archive

STD: sexually transmitted disease

STI: sexually transmitted infections

WHO: World Health Organisation

y: years old

ABSTRACT

Almost every part of our body has coevolved microbial community and as more knowledge of the human microbiota (i.e., community of microorganisms) is acquired, it is becoming evident that microorganisms have a significant impact on our health and well-being. However, an imbalance in this host-microbe relationship can lead to a disease. In the context of human reproductive health, the microbiome (i.e., microorganisms and their genomes) of semen is a field of increasing scientific interest, although this microbial niche has received relatively limited attention compared to other body sites. Despite the evidence of seminal microbiome, a few studies have investigated the sources and acquisition pathways of microorganisms present in the semen. Likewise, the complete influence of the microbes of adjacent sites and its association with male fertility remains unclear. Recent studies have shown that semen harbours its own set of microbes which is polymicrobial and for a low biomass. Further, it has been shown that microorganisms in the semen substantially vary between individuals, suggesting that each has its unique and potentially individual bacterial community profile. Also, certain correlations have been established between the presence of specific bacteria in semen and its quality parameters. Further, bacteria are shared among partners and this bidirectional exchange can influence the microbial make-up of either partner with an implication on their health.

This Doctoral Thesis studies the origin of the seminal microbial communities. The objectives of this Doctoral Thesis were: 1) to investigate the existence of microbes in human testicular samples; 2) to uncover potential sources and routes of microbial colonisation in the semen, and the effect of sterilisation method of vasectomy on seminal microenvironment; 3) to compile available data of microbial niches within couples, to assess the shared microbes within couple, and to determine the potential impact of female

and male reproductive tract microbiomes on couple's health; and 4) to highlight the methodological considerations and provide recommendations for low biomass microbial studies using endometrium as the study model.

Four studies were carried out to address these objectives, with the main findings being: 1) human maturing spermatozoa from testicle biopsies harbour its unique low biomass microbial signature, with a possible role in the functional sperm development, which could be one source of the seminal microbial composition (**Study I**). 2) We found considerable differences in microbial diversity when comparing pre- and post-vasectomy semen samples which suggest that vasectomy influences the seminal microbial composition. Further, semen shares 50% of bacterial communities with urine, underscoring the intricate relationships between anatomically close but functionally distinct niches (**Study II**). 3) During unprotected sexual intercourse, the vaginal and seminal microbes mix and influence each other, forming the "seminovaginal microbiota" which influences the species composition of the couple's reproductive tract, having both beneficial and detrimental effects on the health of couples (**Study III**). 4) The reproductive microbiome field requires clear, reproducible, and well-controlled study design to elucidate the healthy seminal microbiome. When analysing low microbial biomass tissue (as the testicle and semen), systematic control and elimination of possible contamination is crucial to obtain reliable microbiome data over the host information and to minimise misinterpretation of the results. We have proposed a set of guidelines for conducting microbiome studies on low biomass reproductive sites (**Study IV**).

RESUMEN

Casi todas las partes de nuestro cuerpo han coevolucionado con una comunidad microbiana, y a medida que adquirimos más conocimiento sobre la microbiota humana (comunidad de microorganismos), se vuelve evidente que los microorganismos tienen un impacto significativo en nuestra salud y bienestar. Sin embargo, un desequilibrio en esta relación huésped-microorganismo puede llevar enfermedades. En el contexto de la salud reproductiva humana, el microbioma (microorganismos y sus genomas) del semen es un campo de creciente interés científico, aunque este nicho microbiano ha recibido relativamente poca atención en comparación con otras áreas del cuerpo. A pesar de la evidencia del microbioma seminal, pocos estudios han investigado el origen y las vías de adquisición de los microorganismos presentes en el semen. Del mismo modo, la influencia de los microorganismos de sitios adyacentes y su asociación con la fertilidad masculina aún no está clara. Estudios recientes han demostrado que el semen alberga su propio conjunto de microorganismos, que es polimicrobiano y de baja biomasa. Además, se ha observado que los microorganismos en el semen varían considerablemente entre los individuos, lo que sugiere que cada uno tiene su perfil único y potencialmente individual de comunidad bacteriana. También se han establecido ciertas correlaciones entre la presencia de determinadas bacterias en el semen y sus parámetros de calidad. Además, se ha demostrado que las bacterias se comparten entre las parejas y este intercambio bidireccional puede influir en la composición microbiana de cada miembro de la pareja, con implicaciones para su salud.

Esta Tesis Doctoral estudia el origen de las comunidades microbianas del semen. Los objetivos de esta Tesis Doctoral fueron: 1) investigar la existencia de microorganismos en muestras testiculares humanas; 2) descubrir posibles fuentes y rutas de colonización microbiana en el semen, y el efecto del método de esterilización masculina (vasectomía)

en el microambiente seminal; 3) recopilar datos disponibles sobre los nichos microbianos dentro de las parejas, evaluar los microorganismos compartidos y determinar el impacto potencial de los microbiomas del tracto reproductivo femenino y masculino en la salud de la pareja; y 4) resaltar las consideraciones metodológicas y brindar recomendaciones para estudios microbianos de baja biomasa, utilizando el endometrio como modelo de estudio.

Se llevaron a cabo cuatro estudios para abordar estos objetivos, siendo los principales hallazgos los siguientes: 1) los espermatozoides humanos obtenidos de biopsias testiculares albergan su propio microbioma de baja biomasa, con un posible papel en el desarrollo funcional de los espermatozoides, pudiendo ser un origen de la composición microbiana seminal (**Estudio I**). 2) Encontramos diferencias significativas en la diversidad microbiana al comparar muestras de semen antes y después de la vasectomía, lo que sugiere que la vasectomía influye en la composición microbiana seminal. Además, el semen comparte el 50% de las comunidades bacterianas con la orina, lo que subraya las relaciones complejas entre nichos anatómicamente cercanos, pero funcionalmente distintos (**Estudio II**). 3) Durante las relaciones sexuales sin protección, los microorganismos vaginales y seminales se mezclan e influyen mutuamente, formando el "microbioma seminovaginal" que influye en la composición de especies del tracto reproductivo de la pareja, con efectos beneficiosos y perjudiciales para su salud (**Estudio III**). 4) El campo del microbioma reproductivo requiere un diseño de estudio claro, reproducible y bien controlado para determinar el microbioma seminal en individuos sanos. Al analizar tejidos con baja biomasa microbiana (como el testículo y el semen), el control sistemático y la eliminación de posible contaminación son cruciales para obtener datos precisos del microbioma y minimizar la interpretación errónea de los resultados.

Hemos propuesto un conjunto de pautas para llevar a cabo estudios del microbioma en sitios reproductivos de baja biomasa (**Estudio IV**).

GENERAL INTRODUCTION

The human microbiome

Almost every part of our body has coevolved microbial community, i.e., microbiota. In fact, microbes in and on the human body make up 1-3% of our total weight and comprise slightly more cells than our own body (Ursell et al., 2012). In particular, the total number of bacterial cells across the whole body is estimated to be $3.8x10^{13}$, whereas the count of human cells is estimated to be somewhat lower, about $3.0x10^{13}$ (Sender et al., 2016). The human body is primarily inhabited by bacteria, but also viruses (i.e., virome), fungi, archaea, and bacteriophages (Perez-Muñoz et al., 2017). With the advancement of technologies for detecting microbes, more knowledge of the human microbiota is acquired and it is becoming evident that microorganisms have a significant impact on our health and well-being, via producing bioactive molecules both necessary for and harmful to other microbes and for interacting with host cells to regulate and influence our metabolism, physiology, and immune system that eventually shape the overall health and disease resistance (**Figure 1**) (Young, 2017).

The Human Genome Project, launched in 1990, exposed that the human genome comprises only ~20,000 protein-coding genes, which prompted researchers to broaden our knowledge of what constitutes a human and to examine also the microbial communities found in and on the human body (Turnbaugh et al., 2007). As a result, in 2007, the Human Microbiome Project (HMP) was initiated with the goal of enhancing our understanding of microbial communities and their role in human health and disease.

For ages, the microbes in/on our bodies have been largely ignored. First studies of the diversity of the human microbiota were conducted by Antonie van Leewenhoek in 1680s. He studied his oral and faecal microbial communities under the microscope and observed notable dissimilarities in microbial composition between these two niches and between

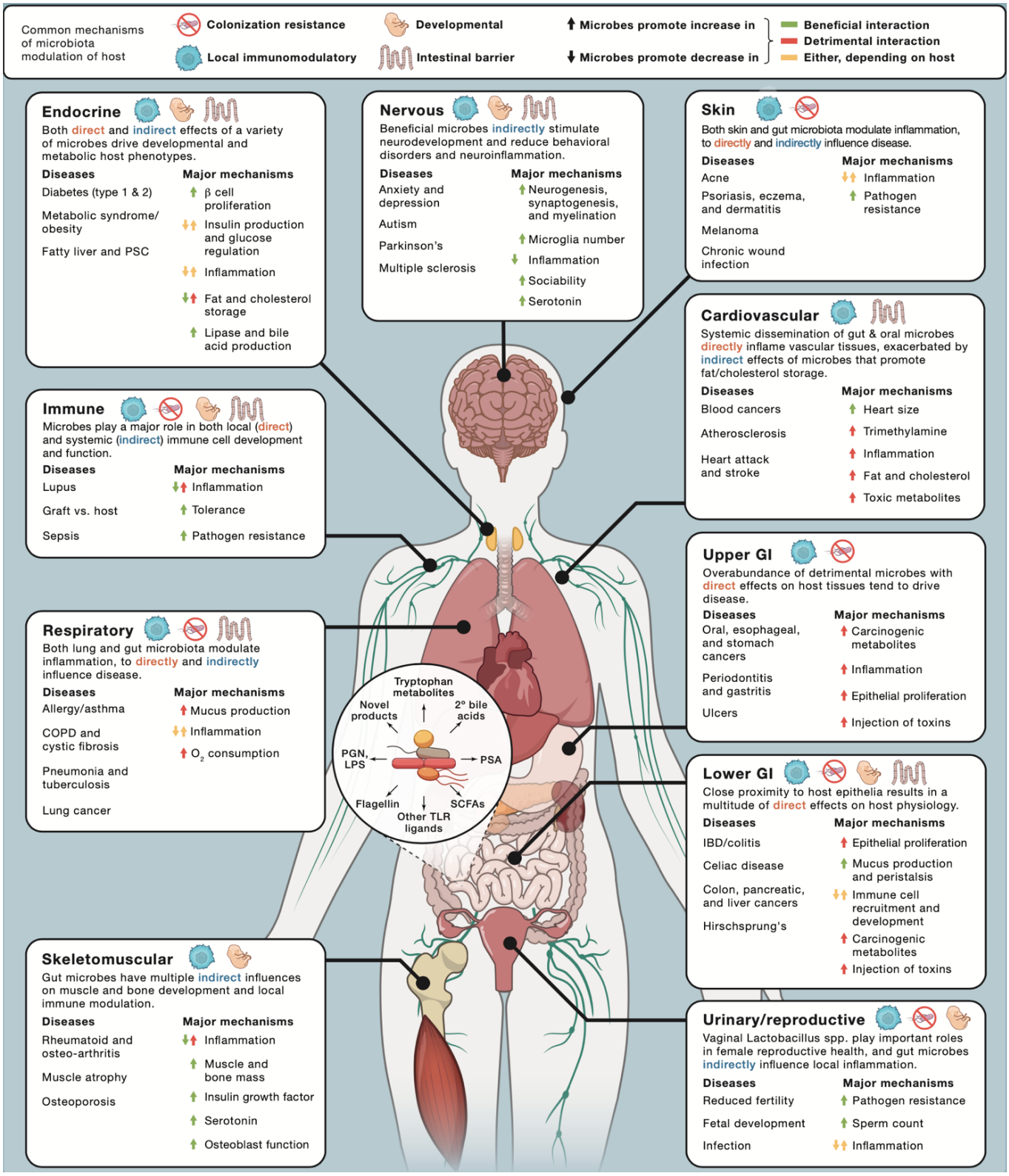

Figure 1. Microbiome influence on host physiology. Depiction of primary organ systems and the role of microbes in preserving homeostasis, as evidenced by germ-free animal models (Hill and Round, 2021). This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5580111505059).

samples with different states of health and disease (Leeuwenhoek, 1684). This fact illustrates that research investigating changes in the microbial composition at different body locations, and between health and sickness, is as ancient as microbiology itself (Ursell et al., 2012). Nonetheless, most human studies have revolved around the disease-causing microorganisms found in individuals, with fewer studies examining the resident microorganisms and their potential benefits (NIH HMP Working Group et al., 2009).

The HMP was pioneered by the NIH Roadmap for Biomedical Research to decipher the "healthy" (i.e., commensal) microbial communities in humans (Turnbaugh et al., 2007). This project was divided into two phases and aimed: 1) to characterise the human microbiome by studying samples from multiple body sites of "healthy" individuals; 2) to determine whether there are associations between changes in the microbiome and health/disease; and 3) to provide both a standardised data resource and new technological methods (NIH HMP Working Group et al., 2009; Turnbaugh et al., 2007). The second phase focussed on dynamic fluctuations in the microbiome and host under different conditions, including pregnancy and preterm birth, inflammatory bowel diseases, and stressors that affect individuals with prediabetes, prompting more questions than it answered concerning the inter-individual variability in terms of microbial composition and its dynamics (Huttenhower et al., 2012; Lloyd-Price et al., 2017; Proctor et al., 2019; The Integrative HMP (iHMP) Research Network Consortium, 2014). The study design of the HMP involved the sampling of five main body parts: the oral cavity, nasal cavity, gut, skin, and vagina in females, resulting in a large-scale analysis of biological samples from 300 United States subjects across 15 body sites applying both 16S ribosomal RNA (rRNA) gene and whole-metagenome sequencing approaches (Huttenhower et al., 2012). This multi-omics study revealed that every single part of the human body is a unique habitat characterised by its own microbial consortia, community dynamics, and

interactions with the host tissue, something previously reported by individually focussed studies (Grice & Segre, 2012). Similarly, it was also confirmed that interpersonal variation was significantly higher than intrapersonal variability. Indeed, the high abundance of microorganisms and personalised composition have led the microbiome, i.e., the genetic material of the microbiota, to be called the "second human genome" (Grice & Segre, 2012). Conclusively, the HMP has paved the way for unravelling the microbial composition and host-microbe interactions in the genitourinary tract in both eubiosis and dysbiosis (i.e., balanced or disrupted state of the commensal microbiome, respectively).

The microbial dynamics, the microbiome diversity, and composition undergo significant changes during the human lifespan (Cho & Blaser, 2012). Immediately after the birth, the microbiome begins to shape gradually, characterised by low diversity and high instability. At this stage, the microbial composition is critically influenced by the type of delivery (vaginal or caesarean) and the method of feeding in the early stages of life, as well as the duration of these practices (Cho & Blaser, 2012). As a child transitions into adolescence, the microbiome starts to stabilise. Yet, it remains highly susceptible to fluctuations due to the developmental (i.e., hormonal, nutritional, and metabolic) changes experienced of this period. Upon reaching adulthood, the microbiome becomes considerably diverse and achieves a stable state, with rare fluctuations typically triggered by ongoing pathologies. Notably, a higher level of microbial diversity is usually indicative of a healthier state. However, as individuals enter old age, the microbial diversity starts to decline, resulting in more similarity between different individuals (Uhr et al., 2019). This transformation of the microbiome across the lifespan, from the neonatal stage to an old age, is an intricate process reflecting the dynamic interaction between our bodies and the microbial world within us.

Microbiome significance in human health

The microbiome plays an essential role in maintaining human health, primarily through interactions with the host's metabolism, immune system, and other physiological processes (Bäckhed et al., 2005). Most commensal bacteria reside in the colon, making it the most exhaustively studied ecosystem within the human body. As suggested by other systems, there is a hypothesised link between the gut microbiome and the male urogenital tract. The theory of the gut-testes axis proposes a connection between the gut microbiome and the male urogenital tract, implying that the microbial environment of the gut can influence testicular function, and thus potentially play a role in male reproductive health and infertility (**Figure 2**) (Leelani et al., 2023). There is considerable evidence suggesting that alterations in the gut microbiome can cause systemic changes and inflammation, as outlined in the "Gut Endotoxin Leading to a Decline in Gonadal Function" (GELDING) theory (Tremellen, 2016). According to that theory, a diet rich in fats and calories can trigger a breakdown in the intestinal mucosal barrier, which results in a leakage of bacterial endotoxins and a subsequent chronic state of low-grade inflammation. This persistent low-grade inflammation can potentially impact the testicular environment, creating an interdependent link. Further, variations in the human gut microbiome have been correlated with changes in systemic sex hormones and spermatogenesis (Magill & MacDonald, 2023). Indeed, several studies have demonstrated that the gut microbiome influences testosterone levels (Matsushita et al., 2022). Specifically, men with higher testosterone levels showed a more diverse gut microbiome, which is generally associated with better health outcomes (Zmora et al., 2019). Congruently, men with low serum testosterone also had an increased abundance of opportunistic pathogens (Liu et al., 2022).

In contrast to the gut, the role of the microbes in other body sites, like the male reproductive tract, is relatively less explored (Altmäe et al., 2019). Yet, emerging evidence suggests a significant role of the microbiome in these niches, highlighting the need for a broader knowledge of the human microbiome beyond the gut.

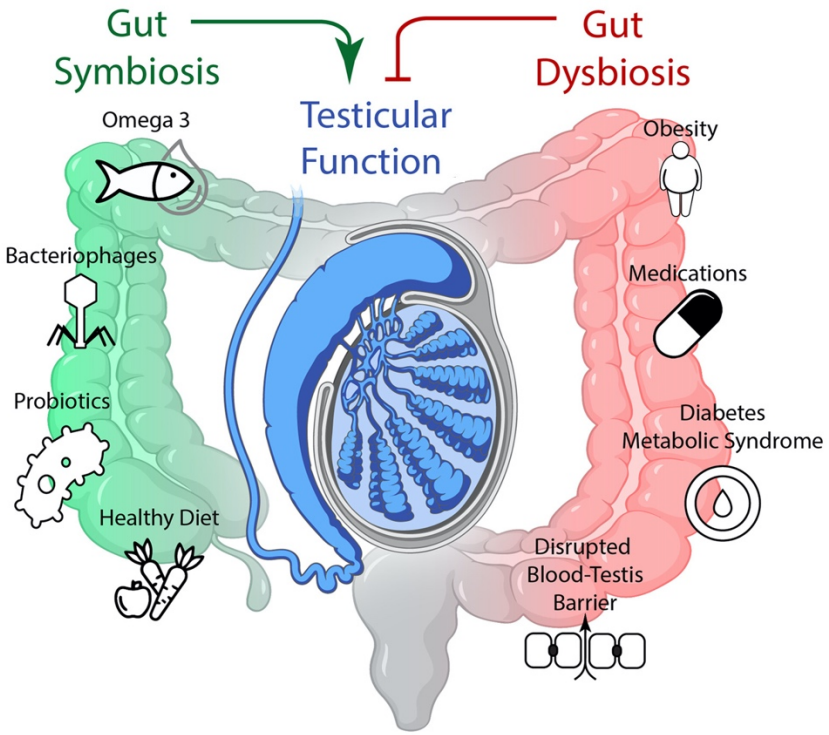

Figure 2. General overview of the gut-testis axis showing some factors that promote gut symbiosis and dysbiosis. In the absence of disease, the gut microbiome exists in a mutually beneficial relationship with the testes, supplying metabolic byproducts from the breakdown of dietary macronutrients. These substances are crucial for the maintenance of the Blood-Testis Barrier and the provision of nutrients necessary for sperm maturation and development. However, dietary shifts or illnesses, such as diabetes or metabolic syndrome, can disrupt the gut microbiome, triggering a cascade effect that culminates in decreased fertility (Leelani *et al.*, 2023). This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5580120142226).

Emerging interest in the microbiome of the male reproductive tract

Historically, the association between male infertility and microbes has been predominantly discussed in the context of acute infections affecting the male reproductive tract (Leelani et al., 2023; Lundy et al., 2020). Early studies on the male genital tract colonisation primarily focussed on recognised genital pathogens, such as *Chlamydia*

trachomatis, *Ureaplasma* spp., and *Mycoplasma* spp. (Baud et al., 2023). These investigations largely relied on traditional bacterial culture methods, leading to a prevailing belief that the semen is minimally populated by bacteria, except in cases of ongoing infections that directly impair fertility. However, in the recent years, the concept of microbes involved in infection and being pathogens has shifted into microbes being commensal and existing in symbiosis with the host in male reproductive tract (Leelani et al., 2023). This shift has been driven by the application of next-generation sequencing (NGS) techniques to characterise bacterial communities colonising the male genital tract. These investigations have corroborated that semen is not sterile, revealing the presence of bacteria not only in men with infertility, but also in men with proven paternity (Altmäe et al., 2019). This is not surprising considering the nature of semen, which is enriched with lipids, saccharides, glycans, inorganic ions, proteins, and peptides (Aalberts et al., 2014; Chiasserini et al., 2015; Drabovich et al., 2014; Jodar et al., 2016; Ronquist et al., 2011), creating an ideal environment for microorganisms.

Thus, human semen harbours its own set of microorganisms which substantially vary between individuals, suggesting that each has its unique and potentially individual bacterial community profile (Hou et al., 2013; Weng et al., 2014). The variability in the microbial composition is shaped by many population and lifestyle factors including age, ethnicity, diet, body mass index (BMI), diseases, therapies (i.e., antibiotic, antifungal, antiviral treatments), administration of pre- and probiotics, stress, physical activity, smoking, and alcohol consumption among others (Altmäe et al., 2019). The seminal microbiome consists mainly of bacteria, making up 71.3% of its composition, but it also hosts small eukaryotes (27.6%) and viruses (1.1%) (Aderaldo et al., 2022). The microbiome predominantly encompasses phyla such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Lundy et al., 2020; Zuber et al., 2023). At genus level,

Corynebacterium, *Lactobacillus*, *Finegoldia*, *Prevotella*, *Staphyloccocus*, *Anaerococcus*, and *Veillonella*, among others, are generally predominant in semen samples (Altmäe et al., 2019).

Male Infertility

Over the recent decades, significant improvements have been made in elucidating factors that influence reproductive health in both males and females. Nevertheless, a considerable number of couples continue to grapple with unexplained infertility, underscoring the need for further research into hitherto unexplored aspects that could contribute to infertility (Leelani et al., 2023). Infertility, defined as the inability to conceive after a year of unprotected intercourse, reportedly affects 10-15% of couples globally (Hanson et al., 2020). Particularly, male factor alone is considered to account for infertility in about a third of these cases and contribute along with female factors in roughly half of all cases (Agarwal et al., 2015). Semen analysis (i.e., seminogram) remains the gold standard for diagnosing and assessing male fertility, classifying semen samples based on several primary parameters such as volume, sperm concentration, motility, and morphology, guided by the World Health Organisation (WHO) reference criteria (World Health Organization, 2021). Despite the insights offered by semen analysis, a significant proportion of male infertility cases remain idiopathic (i.e., with no discernible cause identified), underscoring the complexity and multifactorial nature of the male reproductive health. There are multiple potential causes proposed for idiopathic male infertility, encompassing genetics, epigenetics, proteomics, DNA fragmentation, and microbes (Wagner et al., 2023). Bacteriospermia refers to the condition where bacteria are found in seminal fluid and is clinically recognised when bacteria in semen exceed 1000 colony-forming units (CFU)/ml. This condition is believed to occur in

approximately 6.9-8% of sexually active men (Tvrdá, Ďuračka, et al., 2022). However, this condition increases up to 33% in male population with infertility, although incidence rates can vary from 15% to 70% (Brandão et al., 2021).

Semen dysbiosis and impaired seminal quality

Numerous studies have been conducted to explore the role of seminal dysbiosis and its relationship with male infertility. However, the obtained results are inconclusive, leaving the true impact of bacterial presence on semen quality unclear (Doroftei et al., 2022; Farahani et al., 2020; Magill & MacDonald, 2023). For instance, several studies have observed the association between the seminal microbial richness and diversity and fertility (Amato et al., 2020; Chen et al., 2018; Monteiro et al., 2018), while other studies have detected no associations (Baud et al., 2019; Ma & Li, 2019; Weng et al., 2014).

Despite these inconsistent data, certain correlations have been established between the presence of specific bacteria and semen characteristics (**Figure 3**). For example, seminal hyperviscosity and oligoasthenoteratozoospermia (i.e., low sperm count, poor sperm motility, and abnormal sperm shape) have been associated with the presence of *Neisseria*, *Klebsiella*, and *Pseudomonas*, with a simultaneous reduction of *Lactobacillus* spp. (Monteiro et al., 2018). Interestingly, *Pseudomonas* showed a direct correlation with total motile sperm count and an inverse correlation with seminal pH (Lundy et al., 2021), suggesting the importance of these microorganisms in maintaining the equilibrium within the seminal microenvironment. *Prevotella*, however, has been implicated in disrupting this equilibrium, with increased abundance correlating with an elevated BMI, decreased sperm concentration, abnormal morphology, and defective sperm motility (Baud et al., 2019; Lundy et al., 2021; Okwelogu et al., 2021; Weng et al., 2014). Men experiencing oligospermia (i.e., lower than normal sperm count) exhibit higher quantities of *Prevotella*,

Escherichia, *Lactobacillus*, *Shuttleworthia*, *Serratia*, *Megasphaera*, *Gardnerella*, and *Sneathia* (Contreras et al., 2023). Similarly, *Bacteroidetes* and *Firmicutes* have been widely linked to azoospermia (Alfano et al., 2018; Chen et al., 2018; Hou et al., 2013; Lundy et al., 2021; Monteiro et al., 2018; Weng et al., 2014), where increased levels of *Lactobacillus*, *Enterococcus*, *Corynebacterium*, *Veillonella*, *Gardnerella*, *Ureaplasma*, and *Prevotella* have been characterised (Contreras et al., 2023). A recent shotgun metagenomic study comparing the seminal microbiome of fertile and men with infertility indicated relative differences in the prevalence of the *Propionibacteriaceae* family, along with the *Cutibacterium*, *Rhodopseudomonas*, and *Oligotropha* genera (Aderaldo et al., 2022). Further, research has pointed out specific bacterial strains that negatively impact semen parameters such as *U. urealyticum*, *Mycoplasma hominis*, and *Aerococcus* (Doroftei et al., 2022; Farahani et al., 2020; Magill & MacDonald, 2023; Tomaiuolo et al., 2020; Wang et al., 2022). Also, *Moraxella*, *Brevundimonas*, and *Flavobacterium* in the semen are negatively associated with sperm DNA fragmentation in men with infertility (Garcia-Segura et al., 2022). In contrast, *Lactobacillus* appears to exert a protective effect, as evidenced by its enrichment in control populations with normal semen parameters (Brandão et al., 2021; Contreras et al., 2023; Leelani et al., 2023; Magill & MacDonald, 2023). A greater abundance of *Lactobacillus* in the seminal microbiota was correlated with improved sperm motility and concentration and with normal morphology, probably because it prevents lipid peroxidation (Baud et al., 2019; Moretti et al., 2009; Weng et al., 2014). Remarkably, *Lactobacillus* has been previously associated with sperm elongation and Kruger's strict morphology (Gachet et al., 2022; Weng et al., 2014). Nonetheless, a recent *in vitro* study revealed that adhesion of *Lactobacillus* spp. to sperm cells significantly reduced sperm functions, which could negatively impact reproductive health (Wang et al., 2020). It could be that with a good-

quality semen with abundant spermatozoa the bacterial adhesion does not have noticeable effect on seminal parameters, while it might be pronounced in seminal samples with low spermatozoa counts.

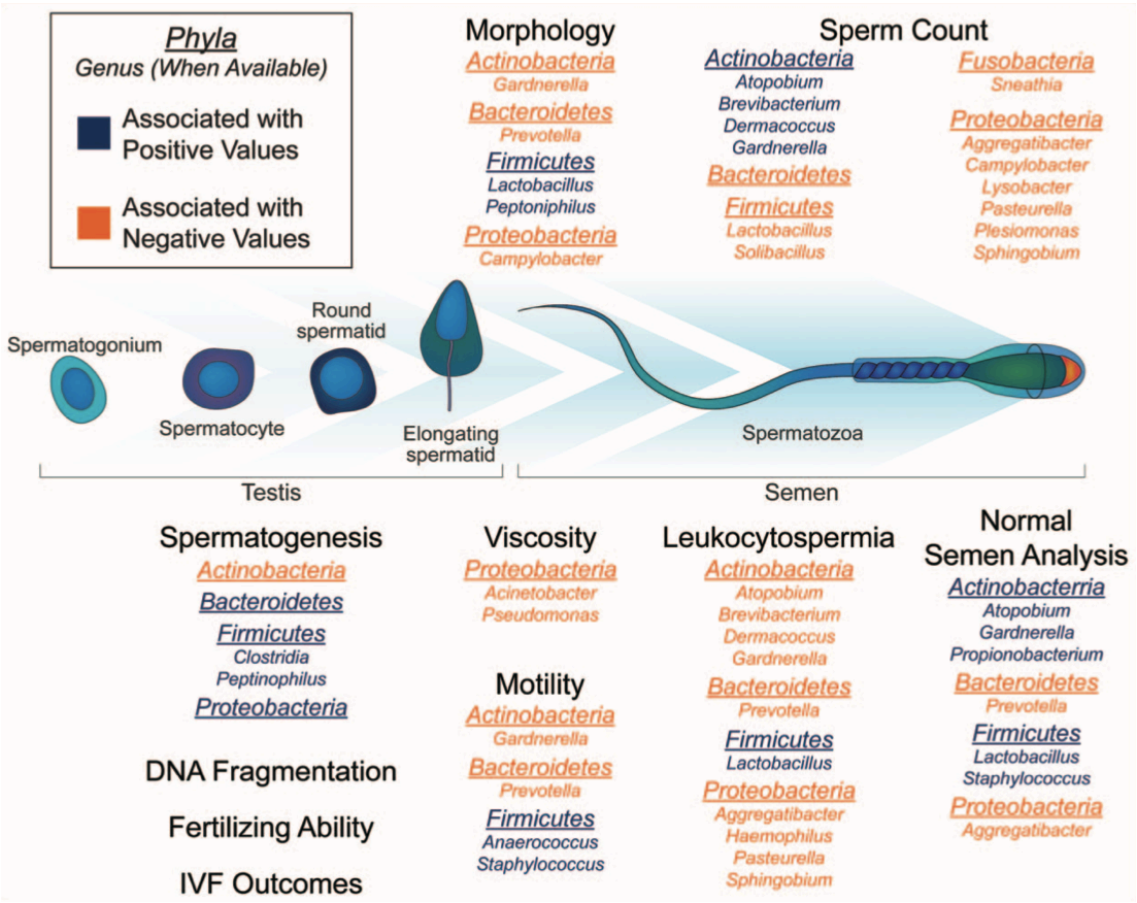

Figure 3. Microbiome influences on sperm production and function. Data gleaned from 16S rRNA-based infertility studies performed on human semen or testicular tissues (Lundy *et al.*, 2020). This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5578391396934).

Bacterial infections have been associated with male infertility, though whether these are the causative factors is often ambiguous (Rowe et al., 2020). An overview of the effects of bacteriospermia on the sperm quality is highlighted in **Figure 4**. First, this bacterial effect on sperm function could potentially be due to a direct sperm-bacteria cellular interactions. One such example is the adhesion of *Escherichia coli* to sperm cells, which can result in sperm agglutination and destruction of the sperm plasma membrane with detrimental effects on sperm motility and ultrastructure (Diemer et al., 1996, 2000).

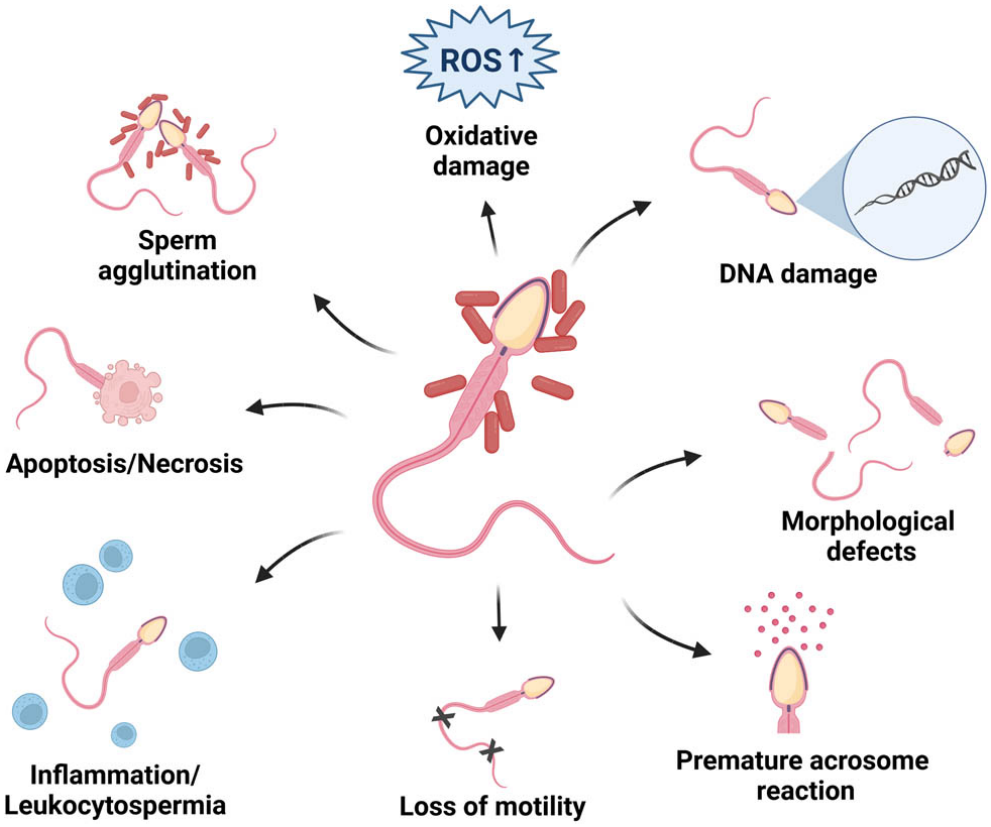

Figure 4. Bacterial impact on sperm quality and function. Common observations include a reduction in sperm motility, changes in sperm morphology, and degeneration of the acrosome. Other frequently documented effects include DNA fragmentation, cell death, and sperm agglutination. Furthermore, bacteriospermia has been found to provoke oxidative stress and stimulate a local immune response (Tvrdá *et al*., 2022). This figure is reproduced under the Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.

Furthermore, bacteria can negatively affect sperm function through the secretion of soluble factors such as porins or lipopolysaccharides (Galdiero et al., 1988). These microbial components can interfere with sperm functionality, possibly by inhibiting macrophage function or inducing overproduction of reactive oxygen species (ROS) (Rowe et al., 2020). Indeed, a link has been suggested between dysbiosis and increased oxidative stress (i.e., production of potentially harmful ROS exceeds the natural antioxidant defence of the body) (Altmäe & Kullisaar, 2022; Tvrdá, Benko, et al., 2022). Notably, bacteria present in semen have been reported to disrupt sperm motility, to cause morphological alterations, impair the acrosome reaction, provoke inflammatory conditions, and cause DNA instability through the overproduction of ROS (**Figure 5**)

(Altmäe & Kullisaar, 2022; Lundy et al., 2020). Further, bacteria-induced disruptions may not stop at physical effects as they could also stimulate the production of antibodies that cross-react with spermatozoa, causing sperm agglutination and immobilisation (Rowe et al., 2020). In summary, the intricate interactions between the bacteria and sperm cells suggest a multifaceted role of the reproductive microbiome in male infertility, covering direct physical effects on sperm, ROS production, immune reactions, and associated oxidative stress.

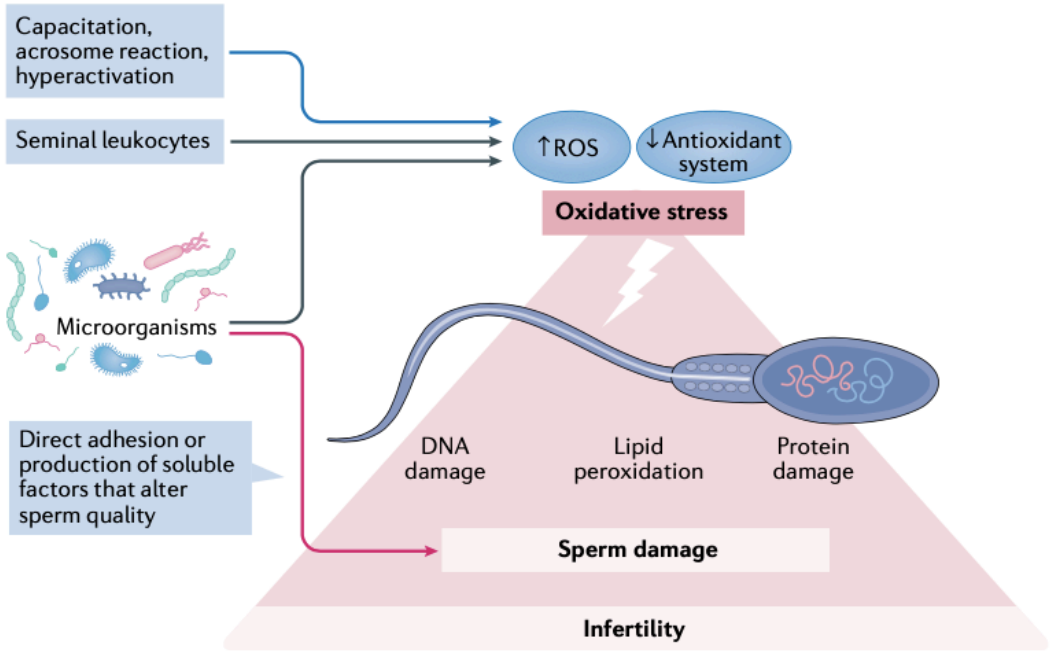

Figure 5. Impact of oxidative stress on sperm quality. Oxidative stress in sperm is the result of an overproduction of harmful reactive oxygen species (ROS) that exceeds the body's natural antioxidant defence, causing cell damage. ROS can be produced both externally by seminal leukocytes and genitourinary microorganisms (black arrows) and internally by the sperm itself (blue arrow). The quality of sperm can be affected by microorganisms, either by inciting host inflammatory responses that trigger ROS production or by directly adhering to the sperm plasma membrane and/or generating sperm-damaging soluble factors (pink arrow). Oxidative stress disrupts various metabolic processes, resulting in various consequences on sperm quality such as elevated sperm DNA fragmentation, reduced sperm motility, and an increased incidence of gene mutations, all of which can contribute to male infertility (Altmäe and Kullisaa, 2022). This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5578411210041).

Although growing evidence indicates that the reproductive microbiomes of both males and females can contribute to fertilisation processes (**Figure 6**). While research into female reproductive microbiomes has made considerable advances, primarily due to an interest in clarifying the microbial transfer between mother and child, comparatively less attention has been given to male reproductive microbiomes. A recent study compared the results after intrauterine insemination (IUI) and found no differences according to sperm diversity or microbiota composition (Amato et al., 2020). On the other hand, higher abundance of *Lactobacillus jensenii* in semen has been associated with a better *in vitro* fertilisation (IVF) rates (Okwelogu et al., 2021). Interestingly, influence of the seminal microbiome on the quality of IVF embryos has been suggested (Štšepetova et al., 2020). Specifically, the presence of *Proteobacteria* and *Corynebacterium* spp. in semen was found to adversely affect the embryo quality. Conversely, *Enterobacteriaceae* were linked to improved embryo quality, as determined by morphological assessments during the cleavage stage (Štšepetova et al., 2020). Although as preliminary, the translation of these findings into clinical practice could bring about potential advances in infertility treatment and management strategies.

The male reproductive tract as a complex ecosystem: origins and sources of seminal microbiome

Semen has its own microbiome, and different parts of the male reproductive system are thought to contribute to its composition. However, there is currently little information about the structure, function and origin of the seminal microbiome, and its importance in male reproductive health.

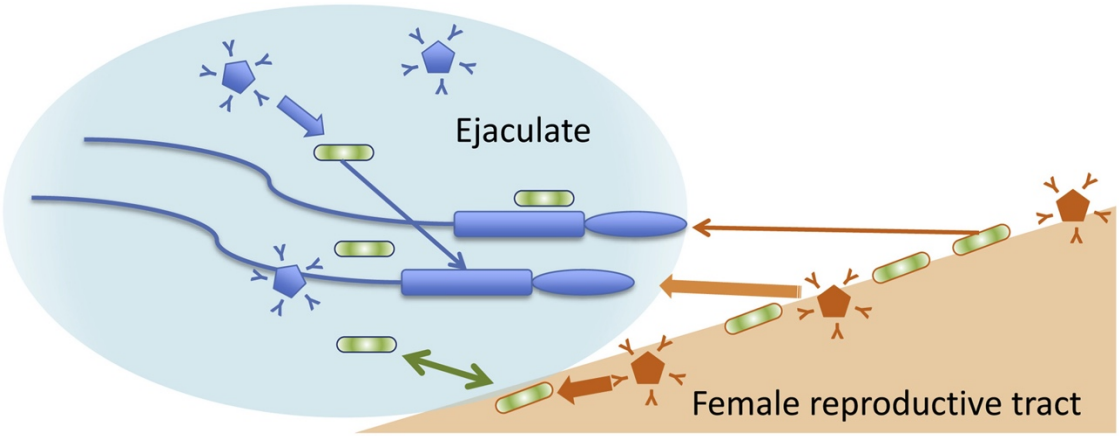

Figure 6. Potential influence of reproductive microbiome spermatozoa and fertilisation dynamics. Microbes present in the semen can affect sperm and, concurrently, become targets for antimicrobial elements (blue pentagons) in the seminal fluid (blue arrows). Likewise, the female reproductive system will immunologically react (orange pentagons) to the local microbes and, post-insemination, to spermatozoa and microbes introduced through ejaculation (orange arrows). Interactions between the microbes in the ejaculate and the vaginal microbiome will also take place (green arrow). The microbial effects are depicted with line arrows, while immunological responses are represented by block arrows (Rowe *et al*., 2020). This figure is reproduced under the Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.

Semen is a fluid produced by the male reproductive system, consisting of sperm and various glandular secretions known as seminal fluid (Mann & Lutwak-Mann, 1981). This slightly alkaline (pH 7.5-8), whitish, viscous liquid is released during ejaculation and serves as the vehicle for spermatozoa during the process of fertilisation (Baskaran et al., 2021). Spermatozoa are produced in the testicles and transported through the vas deferens to the urethra for release (Castillo et al., 2018). As the spermatozoa travel through the vas deferens, they mix with the secretions from other accessory glands, enabling them to mature and capacitate (Castillo et al., 2018). The glandular secretions that make up semen are produced by the epididymis, prostate, seminal vesicles, and bulbourethral glands (Drabovich et al., 2014), providing the nutrients and enzymes necessary for spermatozoa to survive in the vagina and fuse with the oocyte at fertilisation (Castellon et al., 2013). Microbiome has been detected in all the sites of the male reproductive tract and thus the seminal microbiome is not isolated microenvironment but is rather a mixture of different

microbial ecosystems along the male urogenital tract (Zuber et al., 2023). In an earlier culture-based study, 44% of the seminal bacterial species and 58% of those found in the prostate were also detected in the bacterial ecosystem of the urethra (Willén et al., 1996). Some of these species include coagulase-negative *Staphylococcus*, *Corynebacterium* spp., *Peptostreptococcus anaerobius*, *Peptostreptococcus magnus*, *Bacteroides ureolyticus*, and *Prevotella bivia*. Therefore, it has been suggested that the accessory glands and different structures of the male reproductive system contribute to its composition and that semen acquires its microbiome during the sperm's maturation through the spermatic pathways to the outside. Also, an external contribution to the seminal microbiome from the penile skin is plausible. The coronal sulcus features a stable microbiota, circumcision-dependent, where genera like *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, *Anaerococcus*, *Peptoniphilus*, *Prevotella*, *Finegoldia*, *Porphyromonas*, *Acidovorax*, and *Delftia*, among many others, prevail (Price et al., 2010), all of which are present in the seminal microbiome.

The urogenital microbiome, while sharing similarities with the gastrointestinal microbiome via sharing *Lactobacillus*, *Streptococcus*, *Gemella*, *Prevotella*, *Campylobacter*, *Bifidobacterium*, and *Corynebacterium*, displays significant differences that imply a unique contribution from the upper genital tract (Lundy et al., 2021). Specific microbiomes have been associated with the testes, seminal vesicles, and prostate varying between individuals (**Figure 7**). Non-neoplastic testes samples from men with non-metastatic seminoma predominantly contained *Firmicutes* (40%), *Proteobacteria* (35%), *Actinobacteria* (20%), and *Bacteroidetes* (5%) (Alfano et al., 2018). Ten bacterial genera, including *Blautia*, *Clostridium*, and *Prevotella*, were specifically identified in the testicle sperm of men with infertility (Molina et al., 2021). Further, a study describing the microbiome profile in transurethral seminal vesiculoscopy (TSV) samples revealed that

the five most common bacterial phyla were *Firmicutes* (50%), *Bacteroidetes* (22%), *Proteobacteria* (13%), *Actinobacteria* (5%), and *Fusobacteria* (2%), where the top bacterial genera were *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, and *Allobaculum* (Lei et al., 2023). In the prostate, an analysis of benign tissue samples revealed the presence of *Firmicutes* (25%), *Proteobacteria* (8.5%), and *Actinobacteria* (66.5%), with prevalence of *Propionibacterium*, *Corynebacterium*, *Streptococcus*, and *Staphylococcus* genera (Cavarretta et al., 2017). Overall, compiled studies suggest that *Firmicutes* constitutes 50% of the human seminal microbiome, *Proteobacteria* contributes 25%, while the remaining 25% is made up of *Actinobacteria* and *Bacteroidetes* (Altmäe et al., 2019). Factors such as personal hygiene, circumcision, sexual behaviours, and sexual partners can influence these microbiomes. For instance, men without sexual experience were observed to have lower bacterial concentration and diversity compared to those with sexual experience (Mändar et al., 2018). The most prevalent species in a healthy man fluctuate throughout the urogenital tract, which makes it difficult, together with the hard-to-collect invasive biopsies, to fully recognise the origin and dynamics of a healthy seminal microbiome (Altmäe et al., 2019).

Few studies have aimed to investigate the source and acquisition pathways of microorganisms present in semen by comparing the microbial composition of semen samples before and after vasectomy or assessing the disparities between the seminal and urinary microbiomes (Cao et al., 2023; Kermes et al., 2003; Kiessling et al., 2008; Lundy et al., 2021; Suarez Arbelaez et al., 2023). These pioneering studies have underscored changes in the seminal microbial diversity and composition following male sterilisation through vasectomy, suggesting paracrine contribution of upstream anatomic locations such as testis and epididymis (Kiessling et al., 2008; Lundy et al., 2021; Suarez Arbelaez et al., 2023). Similarly, comparative studies between the semen and urine samples have

revealed distinct semen microbial profiles with higher bacterial biomass and modest similarity (~30%) compared to the urinary microbiome (Cao et al., 2023; Kermes et al., 2003; Lundy et al., 2021), suggesting that the microbial composition in these fluids exhibit distinct characteristics and origin.

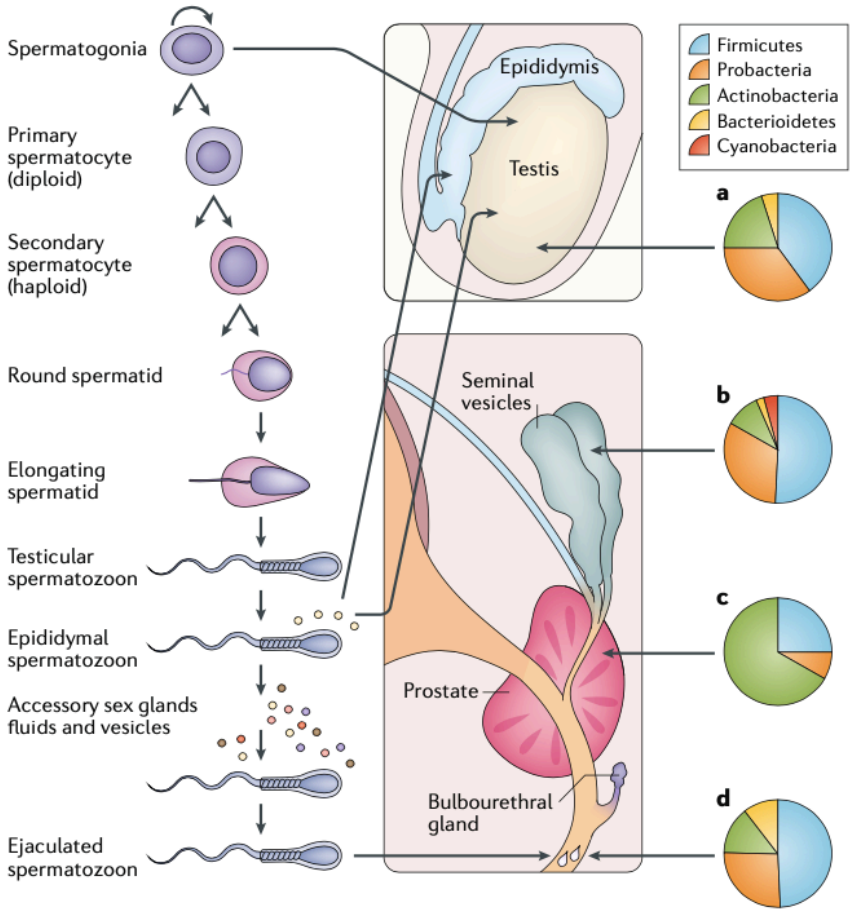

Figure 7. Microbial communities at phylum level along male reproductive tract in healthy individuals. A. Testis. **B.** Seminal vesicles. **C.** Prostate. **D.** Semen. This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5578440171407).

Moreover, it has been observed that the seminal microenvironment can be influenced by interaction with the vaginal microbiome when unprotected sexual intercourse is practiced (Koort et al., 2023; Mändar et al., 2015, 2018). Indeed, the Red Queen evolutionary theory postulates that the microbiomes of seminal and vaginal fluids are expected to reach a certain degree of uniformity, which would be beneficial for sexual reproduction, encompassing aspects like sperm survival or fertilisation from a physiological perspective

(Ma, 2022; Ma & Taylor, 2020). Thus, a connection between the seminal and vaginal, the so called “seminovaginal microbiome”, and the health of the couple and offspring is suggested (Altmäe et al., 2019), nevertheless the molecular mechanisms need to be fully established.

Investigating the composition of the microbiome in the male reproductive tract

Traditionally, investigation of the male reproductive tract microbiome has focussed on the identification of specific bacterial species using culture or PCR methods. However, these approaches often miss non-culturable or unidentifiable bacteria (Almeida & De Martinis, 2019). In contrast, NGS technologies, including marker gene sequencing and shotgun metagenomics, have broadened our understanding of the male reproductive tract microbiome. Through the amplification and sequencing of hypervariable regions of marker gene such as 16S rRNA gene, this method can identify bacterial taxa at various taxonomic ranks, but its discriminatory power is often limited to genus level (Callahan et al., 2017). Similarly, fungal communities are described by sequencing 18S and 28S rRNA genes (Nilsson et al., 2016) and the internal transcribed spacer (ITS) region (Schoch et al., 2012). While the marker gene sequencing methods are extensively used due to their effectiveness, robustness, and low cost, certain limitations such as underestimating microbial diversity and abundance have been noted (Callahan et al., 2021). Despite these shortcomings, marker gene analysis remains the most employed approach and is particularly favoured for studies examining low microbial biomass microbiomes (Knight et al., 2018). Conversely, shotgun metagenomics is based on the sequencing of DNA from all microbial genomes within the sample, providing high coverage of taxonomic composition comprising bacteria, viruses, and eukaryotes with better resolution, potentially offering a species-level classification (Ranjan et al., 2016). Moreover, this

technique can offer insights into the probable functions of microbial communities. However, this approach can be quite costly and biases might be introduced during processes due to the current limitations (especially the annotation part) (Quince et al., 2017).

Conducting studies on the male reproductive tract microbiome has several challenges. First, it should be highlighted that much of the microbial information is focussed on the bacteria rather than the complete microbiota that is also composed of viruses, fungi, and archaea, as bacteria are the most prevalent and also due to the current technical difficulties (Cho & Blaser, 2012). Further, semen samples are a low biomass site, and maintaining sample purity during collection and processing is essential to avoid misleading results (Rowe et al., 2020). Thus, microbiome studies require meticulous design and execution to minimise and account for contamination, especially given the typically non-sterile conditions of assisted reproduction procedures (Contreras et al., 2023; Molina et al., 2021; Štšepetova et al., 2020). Despite these challenges, recent advances in "culturomics" have expanded our ability to culture bacteria from biological samples (Diakite et al., 2020). In culturomics (i.e., high-throughput culturing), a single biological sample is subjected to a variety of culture conditions, such as different temperatures, pH levels, and culture media, to increase the chance of cultivating a broader spectrum of organisms. After growth, the organisms are identified, often by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Lagier et al., 2018). The advent of other "omics" technologies such as metatranscriptomics and meta-metabolomics offer further avenues to explore the intricate relationship between the microbiome and the reproductive health. Collectively, these improvements underscore a promising future for understanding and manipulating the male microbiome to enhance male fertility and health.

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AIMS

General aim

This Doctoral Thesis aims to increase the understanding of the microbiome in male genitourinary system and to elucidate the origin of the seminal microbial communities.

Specific aims

The thesis aims are addressed in four studies:

1) To investigate the existence of microbes in human testicular samples by analysing maturing spermatozoa using 16S rRNA gene sequencing.

2) To elucidate the origin of the seminal microbiome by comparing semen samples collected from the same individuals before and after vasectomy, and urine samples from the same individuals, seeking to uncover potential sources and routes of microbial colonisation in the seminal environment, and the effects of sterilisation method of vasectomy on seminal microenvironment.

3) To compile available data of microbial niches within couples, to assess the shared microbes within couple, and to determine the potential impact of female and male reproductive tract microbiomes on couple's health.

4) To highlight methodological considerations and provide recommendations for low biomass microbial studies.

GENERAL METHODS

This section provides a summary of the methodology applied along this Doctoral Thesis. Detailed explanation of the methodologies can be found in the respective studies. **Table 1** gives an overview of the methods used.

Study I aimed to identify microbes in human testicular samples by analysing maturing spermatozoa using 16S rRNA gene sequencing method and following stringent decontamination protocols together with internal contamination controls at every step throughout the study.

In **Study II**, we set out to explore the potential contribution of the upper reproductive tract together with the urinary microbiome on the seminal microbial composition with the aim to uncover potential sources and routes of microbial colonisation in the seminal environment and to assess the effect of vasectomy procedure on the seminal microenvironment.

In **Study III**, a systematic revision of the literature was performed to gather all publications involving microbiome analysis studies in couples at reproductive age in order to identify the shared microbial composition among genital tracts within the couple.

In **Study IV** aimed to highlight the methodological considerations and propose good practice recommendations for low biomass microbiome studies using endometrial microbiome as the study setting.

Table 1. Methodological overview of the studies included in the Doctoral Thesis

Study	Design	Participants characteristics	Sampling and study material	Main outcome	Methodological approach analyses
Study I	Cross-sectional study	11 men with infertility due to: azoospermia, severe oligoasthenoteratozoospermia, or DNA fragmentation (28-65 years)	Testicular spermatozoa samples from open testicular biopsy	Microbial composition	16S rRNA (V3-4) gene sequencing FASTQ files were denoised using DADA2 Annotation of phylotypes was performed with the RDP database Decontam and microDecon decontamination approaches were applied ANOVA was used to assess differences in microbial signatures between infertility diagnoses and between cell types (FDR)
Study II	Prospective study	82 men who underwent vasectomy (28-49 years)	Semen samples by masturbation and urine samples from the midstream voided urine	Microbial composition and α-, β-diversity indices	16S rRNA (V4) gene sequencing Raw data were demultiplexed with Illumina bcl2fastq2 and imported to QIIME2 FASTQ files were denoised using DADA2 Annotation of ASVs was performed with the SILVA 16S v132_99 database MicroDecon decontamination approach was applied Microbiome α- and β-diversity analyses were performed using R packages Wilcoxon signed-rank test was used for α-diversity

					comparisons, PERMANOVA was permuted for β-diversity testing Differential abundance analysis was performed using ANCOM-BC (FDR)
Study III	Systematic review	Microbiome studies in couples	Semen, penis, and vagina	Shared microbial composition within the couple and microbial α-, β-diversity indices	Search strategy was performed following PRISMA 2020 guidelines Review protocol has been registered in PROSPERO (CRD42022323201) Systematic search of the literature in PubMed, Web of Science, and Scopus Study selection was completed independently by two investigators
Study IV	Methodological and systematic review	Microbiome studies in human endometrium	Endometrium	Methodological considerations and good practice recommendations and microbial composition	Review protocol has been registered in PROSPERO (CRD42020161218) Systematic search of the literature in PubMed, Web of Science, and Cochrane

ANCOM-BC: Analysis of Compositions of Microbiomes with Bias Correction; ANOVA: One-way analysis of variance; ASVs: Amplicon Sequence Variants; DADA2: Divisive Amplicon Denoising Algorithm 2; FDR: False Discovery Rate; NA: Not Available; PERMANOVA: Permutational Multivariate Analysis of Variance; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; PROSPERO: Prospective Register of Systematic Reviews; QIIME2: Quantitative Insights Into Microbial Ecology 2; RDP: Ribosomal Data Project; rRNA: ribosomal ribonucleic acid.

RESULTS AND DISCUSSION

Study I: Assessing the testicular sperm microbiome: a low biomass site with abundant contamination

ABSTRACT

Research question

The semen harbours a diverse range of microorganisms. The origin of the seminal microbes, however, has not yet been established. Do testicular spermatozoa harbour microbes and could they potentially contribute to the seminal microbiome composition?

Design

The study included 24 samples, comprising a total of 307 testicular maturing spermatozoa. A high-throughput sequencing method targeting V3 and V4 regions of 16S rRNA gene was applied. A series of negative controls together with stringent *in silico* decontamination methods were analysed.

Results

Between 50 and 70% of all the detected bacterial reads accounted for contamination in the testicular sperm samples. After stringent decontamination, *Blautia* (*p*-value=0.04), *Cellulosibacter* (*p*-value=0.02), *Clostridium* XIVa (*p*-value=0.01), *Clostridium* XIVb (*p*-value=0.04), *Clostridium* XVIII (*p*-value=0.02), *Collinsella* (*p*-value=0.005), *Prevotella* (*p*-value=0.04), *Prolixibacter* (*p*-value=0.02), *Robinsoniella* (*p*-value=0.04), and *Wandonia* (*p*-value=0.04) genera demonstrated statistically significant abundance among immature spermatozoa.

Conclusions

Our results indicate that the human testicle harbours potential bacterial signature, though in a low biomass, and could contribute to the seminal microbiome composition. Further, applying stringent decontamination methods is crucial for analysing microbiome in low biomass site.

INTRODUCTION

Few, if any, tissues in the human body are entirely sterile, and it is becoming clear that the microorganisms on and in the human body have important functions in health and disease (Power *et al.*, 2017). The Human Microbiome Project has assessed that the urogenital tract accounts for about 9% of the whole human microbiota (NIH HMP Working Group *et al.*, 2009). Nevertheless, little is known about the microbial communities found in the male reproductive tract, and this microbial niche is currently understudied compared with other areas of microbiome research (Altmäe *et al.*, 2019).

With the advancement in technologies for detecting microorganisms, it is now acknowledged that semen harbours a diverse range of bacteria, plays a role in male reproductive health and acts as a medium for the transmission of microbes, with the ability to affect both the couple's and the newborn's health (Altmäe, 2018; Altmäe *et al.*, 2019; Farahani *et al.*, 2020; Osadchiy *et al.*, 2020). Direct sperm–bacteria cellular interactions have been demonstrated, and the possible function of some bacteria in semen could result from these cellular attachments; bacteria seem to firmly attach to the spermatozoon to evade immune responses and to successfully reach the female reproductive tract (Fraczek *et al.*, 2012; Rowe *et al.*, 2020).

The origin and function of the seminal microbes, however, has not yet been established. One-third of the seminal microbes originate from the urethra (Kermes *et al.*, 2003), whereas a substantial part could originate from the upper genital tract. Indeed, the existence of the testicular microbiome was recently presented (Alfano *et al.*, 2018). Alfano *et al*. identified bacterial DNA fingerprints within testicular samples from men with idiopathic non-obstructive azoospermia and found that bacterial dysbiosis was associated with idiopathic non-obstructive azoospermia and complete germ cell aplasia (Alfano *et al.*, 2018). This study provides the first insight into the possible existence of

testicular microbiome and its potential role in functional sperm development (Alfano *et al.*, 2018); nevertheless, no rigorous controlling for contamination was applied. Testicles, like other tissues in the human body (Zheng *et al.*, 2020), harbour limited amount of commensal bacteria, and adequate microbiome identification over the host material is technically challenging and requires well-controlled experiments with rigorous bioinformatic analyses (O'Callaghan *et al.*, 2020).

The aim of the present study was to investigate the existence of microbes in human testicular samples by analysing maturing spermatozoa using 16S ribosomal RNA (16S rRNA) gene sequencing and following stringent decontamination protocols together with internal contamination controls at every step throughout the study.

MATERIALS AND METHODS

Study design and participants

Testicular biopsies from men with infertility were collected at MAR&Gen Assisted Reproduction Clinic, Granada, Spain, when attending for assisted reproductive technology (ART) treatment between September 2014 and April 2016. The study participants presented with azoospermia, severe oligoasthenoteratozoospermia, or DNA fragmentation (**Table 1**). Men with DNA fragmentation underwent testicular biopsy as five or more previous ART cycles had failed. In total, 307 testicular spermatozoa at different maturation stages from 11 men distributed into 24 samples (**Table 1**). All men were screened for sexually transmitted infections (hepatitis B and C, human immunodeficiency virus, cytomegalovirus, syphilis, and *Chlamydia*) and no infections were detected. The study was carried out in accordance with the Declaration of Helsinki, and the procedures was approved by the Ethics Committee of the University of Granada

Table 1. Patient data

Sample[a]	Cell type	Number of cells	Patient	Age (years)	Infertility diagnosis	Sperm concentration (mill/ml)	Progressive motility (%)	Normal morphology (%)	DNA fragmentation (%)
S1	Spermatozoa	5	1	65	Sperm DNA fragmentation	65	73	5	35
S2	Round spermatid	5							
S3	Spermatocyte	5							
S4	Round spermatid	15	2	44	Sperm DNA fragmentation	165	33	3	52
S5		15							
S6	Spermatocyte	15							
S7		15							
S8	Spermatozoa	20	3	32	Sperm DNA fragmentation	20	50	4	45
S9	Spermatocyte	10							
S10	Spermatozoa	5	4	28	Azoospermia	0	-	-	-
S11	Round spermatid	5							
S12	Spermatocyte	5							
S13	Round spermatid	15	5	38	Azoospermia	0	-	-	-
S14	Spermatozoa	10	6	33	Azoospermia	0	-	-	-
S15	Round spermatid	10							
S16	Round spermatid	16	7	52	Azoospermia	0	-	-	-
S17	Spermatocyte	16							
S18	Round spermatid	15	8	41	Azoospermia	0	-	-	-

S19	Spermatocyte	15							
S20	Round spermatid	20	9	45	Azoospermia	0	-	-	-
S21	Non-classified spermatid	15	10	40	Azoospermia	0	-	-	-
S22		15							
S23	Elongated spermatid	20	11	42	Severe oligoasthenoteratozoospermia	0.1	1	0	-
S24		20							

[a] Indicates the name of the sample for downstream analyses.
"-" not assessed

(number 927/2014). All participants gave written consent for the donation of testicular cells for research.

Collection of testicular spermatozoa

Testicular samples were obtained in the air-purificated operating room by open testicular biopsy and were subjected to *in vitro* culture for 5–48 h as previously described (Tesarik *et al.*, 1998). Briefly, an antiseptic was used to clean the scrotum and allowed to dry before the incision for the testicular biopsy. The pieces of testicular tissue were placed in G-GAMETE™ medium (Vitrolife, Gothenburg, Sweden) and disintegrated mechanically by stretching between two microscope slides, followed by repeated aspirations into a 1-ml tuberculin syringe. Large tissue pieces were removed, and the remaining small fragments of the seminiferous tubules were cultured *in vitro*. All cell cultures were carried out in G-GAMETE™ in a water bath set to 30°C. Recombinant human FSH (Puregon, Organon, Oss, the Netherlands) was added at 50 IU/l final activity concentration, and water-soluble testosterone (T-5035) (Sigma-Aldrich, St Louis, MO, USA) was added at a concentration of 1 µmol/l. The cultures were maintained at 30°C.

Testicular cells in *in vitro* cultures could be found both isolated and forming small groups of cells. To achieve the disintegration of the cell clusters, aliquots of all cultures were prepared and incubated with 1000 U/ml of collagenase IV (C-5138) (Sigma-Aldrich Indicated before) at 37°C for 1 h and shaken every 10–15 min during the incubation period followed by recovery in G-MOPS™ medium (Vitrolife). Cells that were not used for clinical procedures were donated for research. In total, 307 testicular spermatozoa at different developmental stages were picked one by one into cell-type specific pools for the present study (**Table 1**). The collection of the 24 cell pool samples from the culture

was carried out under the Olympus IX71 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) using the Tokai-Hit thermal plate (Olympus Corporation, Shinjuku, Tokyo, Japan), the IM–9B microinjector (Narishige Group, Setagaya-ku, Tokyo, Japan) and hatching pipettes (Humagen, Charlottesville, VA, USA). The droplets of cell pools were placed into a 0.2 ml–sterile PCR tube containing cell lysis buffer (with added 3.6 µl Tween–20 [10%], 60 µl dithiothreitol [100 mM] and 6.4 µl RiboLOCK RNase inhibitor [40 U/µl in 30 µl Milli–Q water]), and stored at –80°C for further analysis.

Pre-treatment and DNA extraction

Pre-treatment by bead-beating protocol was carried out to achieve a more efficient bacterial cell lysis. QIAamp *cador*Pathogen Mini Kit was used (Qiagen, Venlo, the Netherlands) following the protocol for difficult-to-lyse bacteria in whole blood or pre-treated tissue by using lysis tubes. As the volume of our starting material was limited (<10 µl), 200 µl of the ATL solution was used. Next, DNA was extracted from the testicular spermatozoa using QIAamp *cador* Pathogen Kit as directed by the manufacturer and the extracted DNA was eluted in 20 µl of AVE solution. Negative controls from the culture media and laboratory reagents were processed in parallel with the 24 testicular sperm samples to control for the possible microbial contamination (**Table 2**).

Sequencing V3 and V4 hypervariable regions of 16S rRNA gene

To characterise the composition of bacterial communities, hypervariable regions V3 and V4 of 16S rRNA gene were amplified by PCR from each sample and sequenced. The primers used were: 5′-CCTACGGGNGGCWGCAG (forward primer) and 5′-GACTACHVGG GTATCTAATCC (reverse primer). All PCRs were carried out in 25 µl

reaction volume containing 12.5 µl 2x KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 µl of each primer (1 µM), and 2.5 µl of extracted DNA (10 ng) under the following cycling conditions using Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific): initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were analysed on 1% (weight/volume) agarose gel electrophoresis in which 1 kb Plus DNA Ladder (catalogue number 10787018) (Thermo Fisher Scientific, Waltham, MA, USA), DNA Gel Loading Dye (6X) (catalogue number R0611) (Themo Fisher Scientific) were used and run under 80 V for 35 min to confirm the amplification of a single product. Amplicons were purified with AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Next, a PCR to index the amplicons was carried out using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). Index PCR conditions using Applied Biosystems 2720 Thermal Cycler were as follows: 95°C for 3 min; eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final extension step of 5 min at 72°C and kept at 4°C. The pooled PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) before quantification. Then, the samples were quantified in a Qubit 4 Fluorometer (Thermo Fisher Scientific). Briefly, the two standards were added in the Qubit 4 equipment (Thermo Fisher Scientific), and the relative fluorescence unit values were checked from 0 to 100 ng/µl. All the samples were measured, including the controls, and after quantification, the samples were normalised at 4 nM using 10 mM Tris pH 8.5 (Sigma-Aldrich). Finally, the samples were denaturalised with 0.2 N NaOH (Sigma-Aldrich), and diluted to a final concentration of 4 pM. The final library was paired-end sequenced at 300-bp using a MiSeq Reagent Kit v.3 on the Illumina MiSeq sequencing system (Illumina.).

Table 2. Negative controls included in the study

Negative controls		DNA amplification	Total number of reads (Decontam)	Total number of reads (microDecon)
	Culture media			
NC1	G-GAMETE™	+	48,349	39,753
NC2	G-MOPS™	+	47,379	36,386
NC3	Cell lysis buffer	+	20,580	14,849
	Laboratory reagents			
NC4	VXL solution[1]	+	66	0
NC5	AW1 solution[1]	+	7	0
NC6	ACB solution[1]	-	-	-
NC7	AVE solution[1]	-	-	-
NC8	2x KAPA HiFi HotStart ReadyMix + primers[2]	+	0	0
NC9	AMPure XP beads + 80% Ethanol + 10 mM Tris pH 8.5[2]	+	0	0
NC10	2x KAPA HiFi Hotstart ready mix + index primers + PCR Grade water[2]	-	-	-
NC11	4 pM PhiX library[2]	-	-	-

[a] The number and the type of negative control used in downstream analyses. After applying the Decontam and microDecon decontamination procedures, the final number of contaminant reads are indicated for each negative control that was taken into account when identifying sperm-specific bacteria and contaminant bacteria in each sample.
[b] DNA extraction kit.
[c] Sequencing library preparation kit.
"-" DNA not amplified

Bioinformatic and statistical analyses

Bioinformatic analysis of the sequencing data was carried out as previously described (Sydor *et al.*, 2020). All fastQ files, generated after sequencing and demultiplexing, were analysed using DADA2 package v.1.10.1 (Callahan *et al.*, 2016) and, as result, a unique table containing all samples with the sequence reads and abundances was generated. Phylotypes were assigned to a taxonomic affiliation based on the naïve Bayesian classification with a pseudo-bootstrap threshold of 80%. Further annotation of phylotypes was performed with the Ribosomal Database Project (RDP) database using the Seqmatch function to define the discriminatory power of each sequence read; annotation was carried

out according to the criteria published previously (Schulz *et al.*, 2018). The resulting phylotype table was filtered to consider only those phylotypes that were present in 50% or more of samples to capture microbes consistently present in the dataset. Microbial communities were analysed at genera phylogenetic rank.

To discern between the true bacterial sequences and potential contaminant DNA, two different decontamination approaches were applied: Decontam v.1.6.0 (Davis *et al.*, 2018) and microDecon v.1.0.2 (McKnight *et al.*, 2019). Given that the characterisation of the low microbial biomass requires *in silico* contaminant removal to ensure that DNA from biological samples can be effectively distinguished from amplified exogenous DNA, the R packages Decontam and microDecon are the most used approaches in the low biomass microbiome studies (Karstens *et al.*, 2019; O'Callaghan *et al.*, 2020).

Decontam

Decontam identifies background DNA contaminants based on their pattern of occurrence in biological versus control samples (Davis *et al.*, 2018). A sequence is classified as contaminant by comparing its associated score statistic P to a user-defined score threshold P^*, where P can be the frequency, prevalence, or composite score (Davis *et al.*, 2018). Specifically, the Decontam score threshold was set to 0.5 to define contaminating phylotypes using the prevalence-based method, as it is recommended for the low microbial biomass environments, e.g., tissue samples (Davis *et al.*, 2018). The prevalence-based method calculates a score for each phylotype (ranging from 0 to 1) that is used by Decontam to distinguish between contaminant and non-contaminants, presenting contaminant phylotypes small scores P (P<0.5). With the score threshold of 0.5, the Decontam package is able to identify 70–90% of contaminant phylotypes (Karstens *et al.*, 2019). Further, the remaining contaminant phylotypes present in low abundance were removed by an additional filtering step, by transforming the testicular

microbial community data set to relative abundances and then setting any phylotype values below 0.1% to zero as described previously (Karstens *et al.*, 2019).

MicroDecon

MicroDecon, a newer decontamination method, is based on the principle that all the samples will receive the same proportions of contamination from a common source and thereby uses the proportions of contaminant sequences in negative controls to identify and remove contaminating reads (McKnight *et al.*, 2019). More specifically, this package identifies a phylotype that is complete contamination, i.e., the "constant", and uses it to calculate the number of reads in each sample that arise from the contamination, and those reads are then subtracted (McKnight *et al.*, 2019). MicroDecon method is suggested to have two advantages over Decontam: first, microDecon treats each sample completely independently and, second, it is not affected by the sample size. MicroDecon can correct phylotypes that occur in both negative controls and real samples, as it is able to remove contaminant reads rather than entire phylotype (McKnight *et al.*, 2019). In the present study, the decon() function was run on its default values, which first decontaminates the data and then applies filtering thresholds to remove residual contamination that should have been removed from all samples but is retained in low numbers in a few samples.

One-way analysis of variance (ANOVA) was used to assess differences in microbial signatures between infertility diagnoses and between cell types. Differences in read counts between testicular cells and internal negative controls were evaluated by Welch's *t*-test. Benjamini and Hochberg correction (false discovery rate, FDR) for multiple testing was applied. FDR p-value<0.05 was considered statistically significant.

RESULTS

In total, 307 testicular spermatozoa at different maturing phases that grouped into 24 samples were analysed, together with 11 negative controls for the microbial profiles. After quality filtering, the total number of paired-end reads and phylotypes in the sperm samples was 3,486,343 and 13,885, respectively. Of the 11 negative controls, six were excluded from further analyses as they did not show any DNA amplification or obtained zero reads after sequencing, i.e., clean controls (**Table 2**). The most contaminated negative controls were the initial *in vitro* culture media, in which the fresh testicular biopsies were placed and cells were cultured (G-GAMETE™ and G-MOPS™). Indeed, it has been recently demonstrated that *in vitro* culture media contains a wide range of microbes (Štšepetova *et al.*, 2020).

Decontamination with Decontam

After applying the contamination correction with Decontam, a total of 1,958,794 paired-end reads were obtained and grouped into 205 phylotypes (**Supplementary Table S1**), with a mean of 81,616 reads and 119 phylotypes per sample.

Contaminant bacteria were detected in all testicular sperm samples, with an average of 45% of contaminant bacterial sequences per sample (ranging from 32–64%) (**Figure 1A**). Decontam analysis identified *Pseudarcicella* (Phy175), *Phascolarctobacterium* (Phy101), Vampirovibrio (Phy98), *Barnesiella* (Phy122), *Alistipes* (Phy170), *Bacteroides* (Phy178 and Phy208), and *Prevotella* (Phy279) as contaminant phylotypes (Decontam score<0.5) (**Figure 1B**), and these taxa were removed from downstream analyses. Further, after abundance filtering, two additional phylotypes with zero reads corresponding to *Bacteroides* genus (Phy932 and Phy973) were identified and removed.

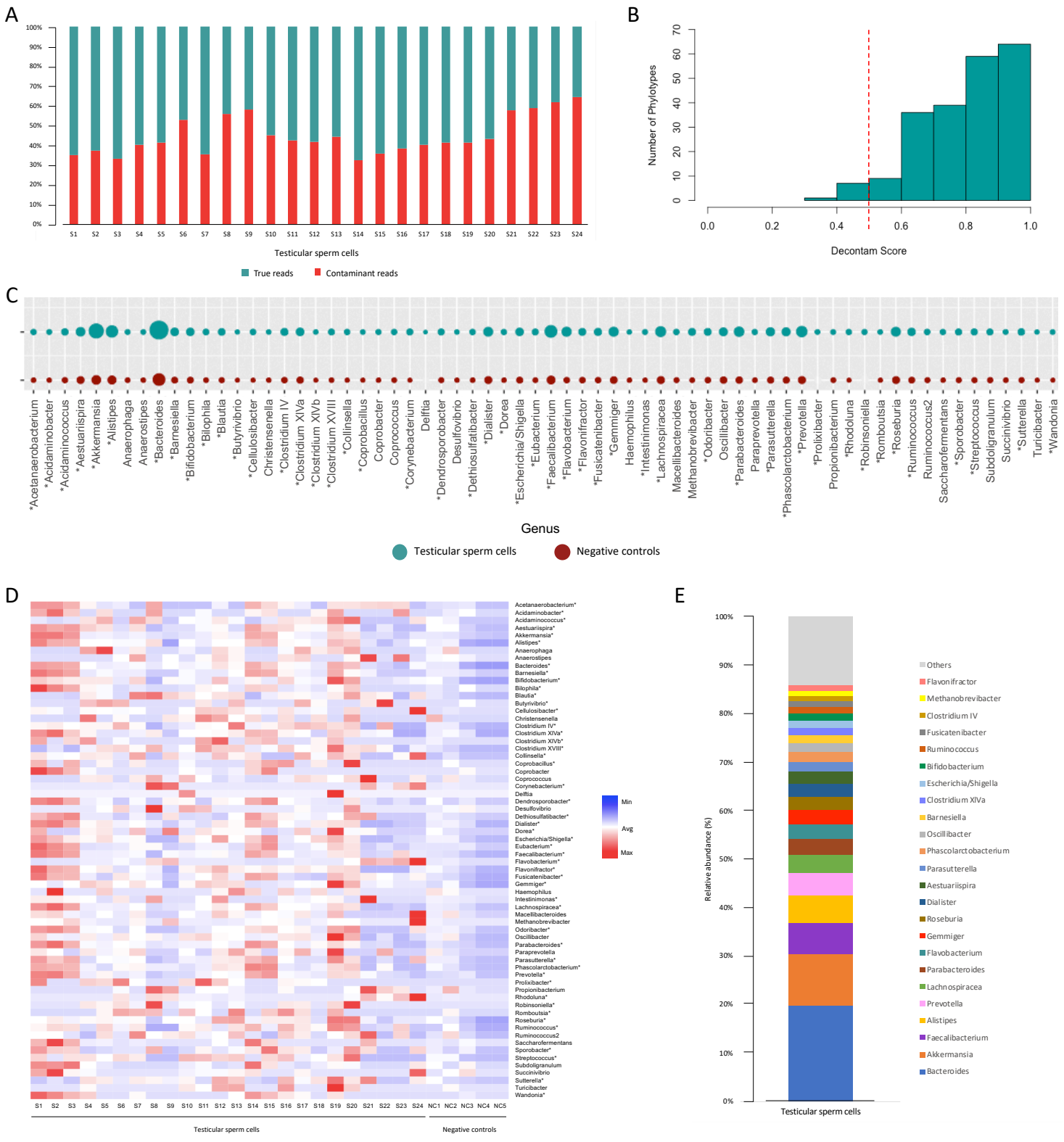

Figure 1. Analysis of microbial sequences in testicular immature spermatozoa using Decontam approach. A. Percentage of true (blue) and contaminant (red) reads obtained in testicular samples. **B.** histogram of prevalence-based scores assigned by Decontam to each phylotype. The x-axis represents the prevalence-based score assigned by Decontam, and y-axis shows the number of phylotypes assigned to a given score. The represented Decontam scores were computed with *IsContaminant* function. The distribution of Decontam scores shows that most of the phylotypes in our samples were assigned high scores (>0.5), suggesting non-

contaminant origin. **C.** average reads of each bacterial genus in testicular samples (blue) versus negative controls (red). The circle size denotes the average reads of each genus. **D.** heatmap illustrating the number of reads at genus level in each testicular sample and negative control. "Average" indicates average score that has the same raw value as the row mean, "Maximum" indicates maximum score that has standard deviation (SD) above the row mean, and "Minimum" denotes minimum score that has SD below the row mean. **E.** the "clean" bacterial composition in testicular samples at genus level. Genera with abundance less than 1% were grouped as "others". * indicates genera that differed statistically (p-value<0.05) (Supplementary Table 2) in the number of reads between the testicular sperm samples versus negative controls.

Although negative controls presented similar bacterial profile to testicular samples, the number of the reads differed significantly (**Figure 1C** and **1D** and **Supplementary Table S2**). The testicular samples contained 66 genera and the negative controls 63 genera (**Figure 1C**). Genera not identified in negative controls included *Delftia, Prolixibacter* and *Robinsoniella*. Sequencing of testicular maturing spermatozoa revealed that the dominant genera included *Bacteroides, Akkermansia, Faecalibacterium, Alistipes*, and *Prevotella* (**Figure 1E**).

Decontamination with microDecon

Decontaminated output of microDecon analysis contained 976,323 paired-end reads grouped into 171 phylotypes (**Supplementary Table S1**), with a mean of 40,680 reads and 96 phylotypes per sample. This method detected and removed contaminant reads in all testicular sperm samples, rather than assigning an entire phylotype as contaminant, which resulted in an average of 72% of contaminant bacterial sequences per sample (ranging from 65–78%) (**Figure 2A**).

Negative controls and testicular samples presented similar bacterial profiles; however, the number of the reads differed statistically, being higher in sperm samples (**Figure 2B** and **2C** and **Supplementary Table S2**). With microDecon approach, 60 genera in testicular sperm and 59 in negative controls were detected (**Figure 2B**). *Robinsoniella* was the only

genus not identified among negative controls. The dominant genera detected in the immature spermatozoa included *Bacteroides, Akkermansia, Faecalibacterium, Alistipes*, and *Flavobacterium* (**Figure 2D**).

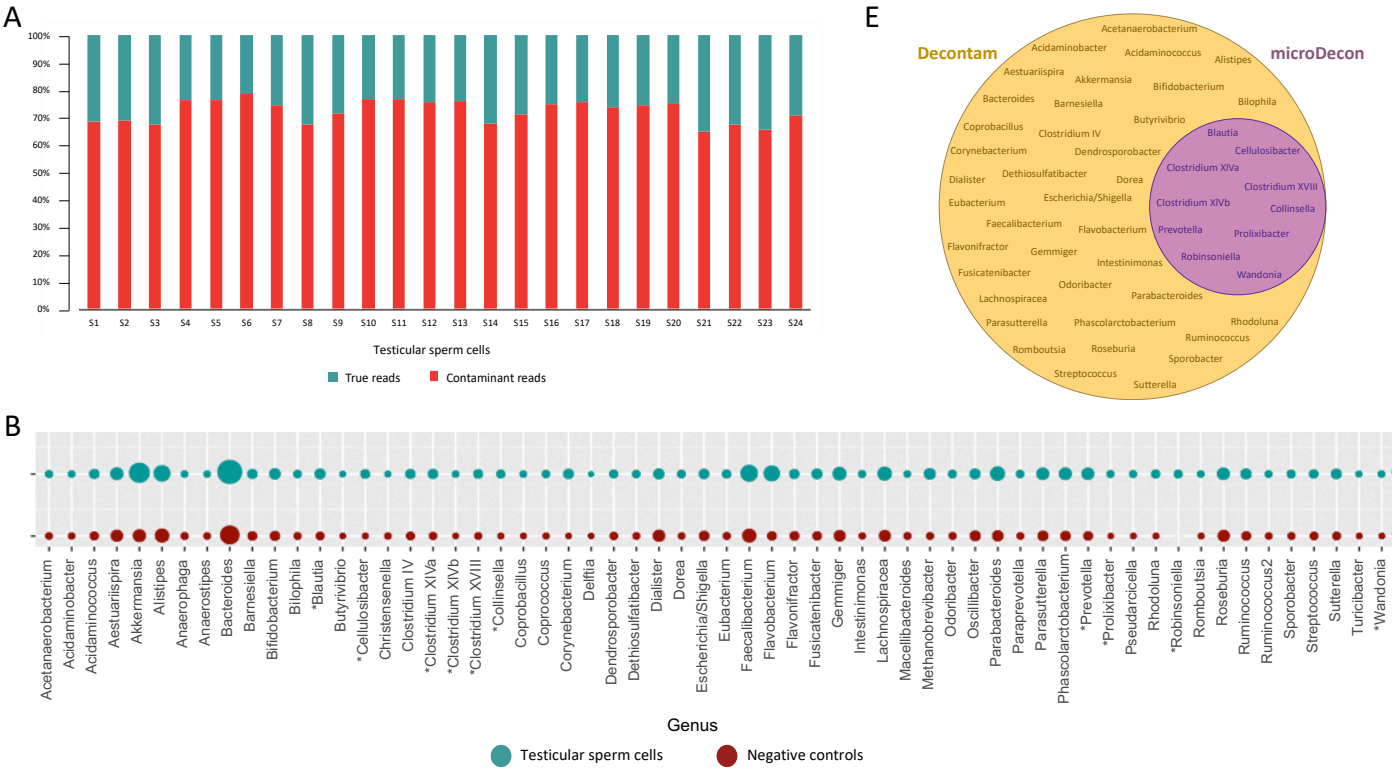

Figure 2. Analysis of microbial sequences in testicular immature spermatozoa using microDecon approach. A. Percentage of true (blue) and contaminant (red) reads obtained in testicular samples. **B.** average reads of each bacterial genus in testicular samples (blue) versus negative controls (red). The circle size denotes the average reads of each genus. **C.** heatmap illustrating the number of reads at genus level in each testicular sample and negative control. "Average" indicates average score that has the same raw value as the row mean, "Maximum" indicates maximum score that has standard deviation (SD) above the row mean, and "Minimum" denotes minimum score that has SD below the row mean. **D.** the "clean" bacterial composition in testicular samples at genus level. Genera with abundance less than 1% were grouped as "others". **E.** significantly more abundant genera in testicular samples versus negative controls in both decontamination approaches ($P<0.05$). *Blautia, Cellulosibacter, Clostridium* XIVa, *Clostridium* XIVb, *Clostridium* XVIII, *Collinsella*, *Prevotella*, *Prolixibacter*, *Robinsoniella*, and *Wandonia* are considered to be testicle sperm-specific bacteria.
* indicates genera that differed statistically (p-value<0.05) (Supplementary Table S2) in the number of reads between testicular sperm samples versus negative controls.

To compile the contamination results, the number of detected DNA sequences in the negative controls (contaminant reads) was lower than in the biological samples. After subtracting these contaminant reads from the testicular samples (applying Decontam and microDecon methods), a microbial signature in the testicular cells was identified. Significantly more abundant genera were found in the testicular samples compared with controls after applying both decontamination approaches (**Figure 2E**).

No statistically significant differences in microbiome profiles were detected between individuals and between testicular spermatozoa in different developmental stages (**Supplementary Figure S1** and **Supplementary Figure S2**).

DISCUSSION

The present study findings help to unravel the microbial composition in the testicle; however, it seems to be a low microbial biomass site. Microbiome analysis of a low microbial biomass site requires specific focus on combating host and laboratory reagent microbial contamination to identify true bacterial sequences (Karstens *et al.*, 2018, 2019; Eisenhofer *et al.*, 2019; Stinson *et al.*, 2019; Weyrich *et al.*, 2019; O'Callaghan *et al.*,

2020; Molina *et al.*, 2021). In the present study, internal negative controls were used throughout all the experimental steps and additionally applied rigid *in silico* decontamination methods for unravelling the non-contaminant microbiome in the testicular sperm samples. Altogether, 10 bacterial genera were identified as testicle sperm specific. These included *Blautia* (phylum *Firmicutes*)*, Cellulosibacter* (*Firmicutes*)*, Clostridium* XIVa (*Firmicutes*), *Clostridium* XIVb (*Firmicutes*), *Clostridium* XVIII (*Firmicutes*), *Collinsella* (*Actinobacteria*), *Prevotella* (*Bacteroidetes*), *Prolixibacter* (*Bacteroidetes*), *Robinsoniella* (*Firmicutes*), and *Wandonia* (*Bacteroidetes*). The detected bacteria *Blautia, Clostridium*, and *Prevotella* have also been identified in previous studies among the seminal samples (Weng *et al.*, 2014; Altmäe *et al.*, 2019; Campisciano *et al.*, 2020; Štšepetova *et al.*, 2020; Yao *et al.*, 2020), demonstrating that the most abundant bacteria in the testicular sperm samples are also present in the semen and supporting the possible contribution of the upper genital tract microbes to the downstream seminal microbiome composition. Interestingly, *Prevotella* was identified in over 90% of our testicular samples. *Prevotella* genus has been associated with low-quality semen when analysing semen samples from humans (Jarvi *et al.*, 1996; Nguyen *et al.*, 2014; Weng *et al.*, 2014; Baud *et al.*, 2019; Campisciano *et al.*, 2020; Farahani *et al.*, 2020; Yang *et al.*, 2020), suggesting that species within *Prevotella* could contribute to the spermatogenesis defects and male infertility (Ding *et al.*, 2020; Yang *et al.*, 2020). The pioneering study of the testicular microbiome (Alfano *et al.*, 2018) did not present their results on bacterial genus level; therefore, our study results on specific testicular bacteria are not comparable, whereas, on phylum level, our identified phyla were also reported in the previous study.

Another important result of our study is that contamination comprised 50–70% of all the detected bacterial reads in our testicular cell samples, supporting the hypothesis that assisted reproductive technology is not carried out in sterile conditions (Štšepetova *et al.*,

2020), and highlighting the importance of controlling for the possible contaminants when dealing with low microbial biomass tissue. Indeed, it has been demonstrated that contaminant microorganisms, specifically the contaminants arisen before amplification, can dominate the composition of low-microbial-biomass samples, which could lead to inaccurate data interpretation (Salter *et al.*, 2014; Glassing *et al.*, 2016).

In the present study, all contaminating steps in analysing microbiome were controlled for; however, the study has limitations that should be highlighted. One limitation is the analysis of cultured spermatozoa instead of untreated cells, which might have favoured the growth of some bacteria. The culturing media, however, were treated as negative controls, and the results were rigorously controlled for a possible contamination arising from this step. Also, inclusion of positive control (mock microbial community) would have helped to assess the amplification efficiency and the possible cross-contamination during sample processing. Furthermore, although we analysed microbial composition of testicular sperm samples from men with infertility, whose testis microbiome could be altered, knowledge of the healthy commensal microbiome in the human testicles was lacking.

In conclusion, our study results indicate that the testicle harbours its unique low biomass microbial signature, with a possible role in functional sperm development, and could be one source of the seminal microbial composition. Nevertheless, further research is required for assessing the potential effect of short microbial DNA fragments as determinants of spermatogenesis and male reproductive health outcomes. We also conclude that when analysing low microbial biomass tissue, such as the testicle, systematic control and elimination of possible contamination is crucial to obtain reliable microbiome data over the host information and to minimise misinterpretation of the results.

SUPPLEMENTARY MATERIAL

Supplementary files may be found online in the Supplementary materials section: https://www.rbmojournal.com/article/S1472-6483(21)00305-9/fulltext

DATA AVAILABILITY

Sequence data of all testicular spermatozoa and negative control samples have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject ID PRJNA643898.

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Study II: Unravelling the origin of the seminal microbiome: comparative analysis of semen and urine samples before and after vasectomy

ABSTRACT

The semen harbours a polymicrobial community; however, the origin of the seminal microbiome has not yet been clearly established. One-third of the seminal microbes originate from the urethra, whereas a considerable part could originate from the upper genital tract. Similarly, male reproductive organs, such as prostate, seminal vesicles, and testicles contain its own microbiome. Recent pioneering studies on limited sample size indicate that vasectomy procedure alters the seminal microbiome, suggesting a testicular or epididymal microbial origin. This cohort study included 82 men who were planning to undergo vasectomy and provided paired semen and urine samples before and after the vasectomy. The seminal microbiome was analysed by sequencing the V4 hypervariable region of the 16S rRNA gene. We found that vasectomy influences the seminal microbial composition and that the semen shares 50% of bacterial communities with urine, altogether indicating paracrine effects of the genitourinary system on seminal microenvironment. Our study provides new insight into the origin of seminal microbes, suggesting that part of the seminal microbiome could originate from the testicular and urinary environment. Furthermore, we confirm the effect of vasectomy procedure on seminal microenvironment, which could have a short- and long-time effect on male urogenital health.

INTRODUCTION

The human microbiota, consisting of trillions of microorganisms inhabiting various anatomical sites, has emerged as a crucial player in human health and disease (Rowe *et al.*, 2020). Broad research has shed light on the diverse microbial communities residing in the gut, oral cavity, and urogenital tract, influencing numerous physiological processes, and contributing to overall wellness (Gilbert *et al.*, 2018; Altmäe *et al.*, 2019). However, despite its significance, the exploration of the microbiome (i.e., microorganisms and their genomes) in certain human niches remains unexplored. Especially, the seminal microbiome has received relatively limited attention compared to other body sites (Altmäe *et al.*, 2019).

Understanding the seminal microbiome and its origin is essential as it may play a pivotal role in the male reproductive health (Lundy *et al.*, 2021; Altmäe and Kullisaar, 2022; Suarez Arbelaez *et al.*, 2023). Semen, traditionally considered as a sterile fluid, has been recognised as an emerging niche for microbial colonisation (Venneri *et al.*, 2022; Contreras *et al.*, 2023). Accordingly, investigating the seminal microbiome has gained attention due to its potential implications in male fertility, reproductive disorders, and overall reproductive health. Nevertheless, the role of the semen microbiome has not been completely elucidated, the studies indicate its association with seminal quality and its influence on inflammation and immune responses (Altmäe *et al.*, 2019).

Despite growing interest in the seminal microbiome, a few studies investigated the sources and acquisition pathways of microorganisms present in semen, by comparing the microbial composition of semen samples before and after vasectomy or assessing the disparities between seminal and urinary microbiomes (Kermes *et al.*, 2003; Kiessling *et al.*, 2008; Lundy *et al.*, 2021; Cao *et al.*, 2023; Suarez Arbelaez *et al.*, 2023).

These pioneering studies have highlighted alterations in the seminal microbial diversity and composition following male sterilisation through vasectomy, suggesting paracrine contribution of upstream anatomic locations such as testis and epididymis as contributors to the seminal microbiome (Kiessling *et al.*, 2008; Lundy *et al.*, 2021; Suarez Arbelaez *et al.*, 2023). Likewise, comparative studies between semen and urine samples have revealed distinct semen microbiome with modest similarity (~30%) to the urinary microbiome (Kermes *et al.*, 2003; Lundy *et al.*, 2021; Cao *et al.*, 2023), suggesting that the microbial composition in these fluids exhibit distinct characteristics and origin. Indeed, seminal microbiome could partly originate from the upper genital tract as existence of microorganisms in the testis (Alfano *et al.*, 2018; Molina *et al.*, 2021a) and prostate (Cavarretta *et al.*, 2017; Yow *et al.*, 2017; Feng *et al.*, 2019; Jain *et al.*, 2020; Wu *et al.*, 2020) have been identified.

On the other hand, vasectomy is a common procedure for sterilisation, which prevalence in Europe and North America is approximately 10%, with certain countries reaching 20% among reproductive aged men (Jacobstein, 2015; Degraeve *et al.*, 2022). This procedure causes changes in semen viscosity, pH, and prostaglandin levels that affect inflammation in addition to other functions (Brummer, 1973; Nikkanen, 1979). These oscillations in seminal characteristics could in part be the result of microbial alterations, as microbiome is an important regulator of inflammation and autoimmunity (Ding *et al.*, 2020). Therefore, changes in the microbial composition following vasectomy could lead to dysbiosis in the seminal microbiome which might have long-term effects on male health (Suarez Arbelaez *et al.*, 2023).

In the current study, we set out to explore the seminal microbiome fluctuations induced by vasectomy by analysing paired seminal and urine samples collected from the same individuals before and after vasectomy. We aimed to investigate the potential contribution

of the upper reproductive tract together with the urinary microbiome to the microbial composition in semen to uncover potential sources and routes of microbial colonisation in the seminal microenvironment.

MATERIALS AND METHODS

Study population

The study was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and the legally enforced Spanish regulation, which regulates the clinical investigation of human beings (RD 223/04). All procedures were approved by the Ethics Committee of the Investigación Biomédica de Andalucia (ref. CEIM/CEI 0463-M1-18r). Written informed consent was obtained from all subjects prior to inclusion.

Eighty-two men who were planning to undergo vasectomy were recruited at the University Hospital Virgen de las Nieves, Granada between February 2021 and October 2022. All participants donated urine and semen samples before the vasectomy and 3 months after the procedure with confirmed azoospermia in the semen analysis. In the case of presence of spermatozoa, the sample was repeated 3 months later with confirmed azoospermia. No preoperative or postoperative antibiotics were prescribed.

Participants were informed that they should stay sexually abstinent for 3-5 days. All semen samples were self-collected at the Hospital by masturbation into a sterile polypropylene 120ml-container (DELTALAB, Barcelona, Spain). Patients performed hand sterilisation and collected semen sample after washing the glans penis with soap and water, and after urinating. Samples were immediately provided to andrology lab technicians for processing. Before liquefaction and routine semen analysis, 200µl-aliquot from each semen sample was placed in a cryovial (VWR®, part of Avantor, Barcelona,

Spain), snap-frozen in the gas phase of liquid nitrogen and stored at -80°C for further analysis.

Urine samples were collected from the midstream into a sterile polypropylene 120ml-container (DELTALAB) prior to the semen sampling. Next, 3 ml were pipetted in 1 ml of nucleic acids' stabiliser medium (eNAT® 608CS01R, COPAN Italia, Brescia, Italy), kept at room temperature max 6 hours, and stored at -80°C for further analysis.

Additionally, participants completed a questionnaire that included demographic characteristics, lifestyle factors, and sexual activities. BMI was calculated from the self-reported weight and height data.

Semen analysis

The rest of the sample was taken for the assessment of the sperm parameters (i.e., sperm volume, concentration, and total progressive mobility) according to the WHO guidelines (World Health Organization, 2021) and the semen analysis methodology checklist (Björndahl *et al.*, 2022).

DNA extraction

For microbiome analysis, genomic DNA was extracted from semen samples using the QIAamp DNA Microbiome Kit (QIAGEN, Venlo, The Netherlands) and the QIAamp UCP Pathogen Mini Kit (QIAGEN) for urine samples, following the manufacturer's instructions. The purity, quality, and yield of the extractions were determined by measuring the A260/A280 and A260/A230 ratios with the NanoDrop ND1000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration was quantified by fluorimetry with Qubit 4 (Thermo Fisher Scientific) and normalised.

Negative and positive controls were included and processed along with the biological samples to monitor the potential microbial contamination. Negative controls included sample collection controls for each tissue source, DNA extraction (e.g., reagent) controls, library preparation controls, and sequencing controls (**Supplementary Table S1**). Positive controls included the ZymoBIOMICS (Zymo Research, Irvine, CA, USA) mock community standard.

Analysis of 16S rRNA gene sequencing

Seminal and urinary microbiomes were profiled by amplifying the bacterial-specific V4 hypervariable region of the 16S rRNA gene and sequencing. The primers used were 515F (5'-GTGYCAGCMGCCGCGGTAA) and 806R (5'- GGACTACNVGGGTWTCTAAT). All PCRs were performed in 25 µl reaction volume containing 12.5 µl 2x KAPA HiFi Hotstart ready mix (KAPA Biosystems, Wilmigton, MA, USA), 5 µl of each primer (1 µM), and 2.5 µl of extracted DNA under the following cycling conditions using Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific): initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 90 s, with a final extension at 72°C for 10 min. A quality control was performed using 2% agarose gel electrophoresis to verify that each sample had been amplified. The expected amplicon size was around 380 bp. Each sample was quantitated separately by fluorimetry with Qubit 4 (Thermo Fisher Scientific) and pooled equimolarly with an optimal amount of 50 ng per sample. PCR products were first purified by column using MicroElute Cycle Pure Kit (Omega Bio-tek, Norcross, GA,

USA) and next with AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). To check that there were no primer residues and that the library size was as expected, a quality control was performed with an HS bioanalyser (Agilent technologies, Santa Clara, CA, USA). Illumina Nextera library preparation was performed according to the manufacturer's specifications, combining PhiX phage (20%) with the amplicon library to give diversity to the run. The final library was paired-end sequenced (2 × 300 bp) using a MiSeq Reagent Kit v.3 on the Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

Bioinformatic and statistical analyses

Raw data were demultiplexed with Illumina bcl2fastq2 Conversion Software (v2.20) and imported to QIIME2 software (v.2022.11) with a PairedEndFastqManifestPhred33 input format. Divisive Amplicon Denoising Algorithm 2 (DADA2) was used for the denoising step. Low-quality regions were trimmed considering a quality score below 25 to create high quality forward and reverse reads, using the "q2-dada2" function. Taxonomy assignment of amplicon sequence variants (ASVs) was performed using the "classify-sklearn" function against the SILVA 16S v.132_99 database, along with a similarity threshold of 99%. Microbial taxa were aggregated to phylum and genus level in further analysis.

The resulting ASV tables were decontaminated based on proportions of contaminant sequences in negative controls, identifying and removing contaminating reads from biological samples. The decontamination approach was performed in R (v.4.2.2) under RStudio (v.2022.12.0+353). In particular, the "decon()" function from microDecon package was run on its default values. Additionally, the decontaminated tables were

filtered to consider only those taxa that were present in ≥30% samples included in each comparison group in order to capture microorganisms consistently present in the niches.

Two sets of analyses were performed. The first compared paired pre- and post-vasectomy microbial profiles in semen samples. Paired sample analysis aims to mitigate the impact of population and lifestyle factors on the microbial composition outcome while providing a more comprehensive understanding of the specific microbiome changes associated exclusively with vasectomy (Suarez Arbelaez *et al.*, 2023). The second analysis compared paired seminal and urinary microbiomes to assess the possible microbial contribution of the urinary tract to the seminal environment.

Microbiome diversity analyses were also conducted under RStudio using phyloseq, vegan, microviz, and ggplot2 R packages. Within-sample microbiome diversity (i.e., α-diversity) was estimated by Shannon diversity index and richness (i.e., number of microbial taxa), using the "diversity" and "specnumber" functions from the vegan package. Between-sample microbiome dissimilarity (i.e., β-diversity) was visualised using nonmetric multidimensional scaling (NMDS) ordination, based on the Bray Curtis distance. For α-diversity comparisons in paired data, Wilcoxon signed-rank test was used for significance testing with the function "wilcox.test()". For β-diversity testing, PERMANOVA was permuted using the "adonis2" function from vegan package. Differential abundance analysis was performed on those bacterial genera present at least in ≥30% samples included in each comparison group using an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020) from the ancombc2 R package. All *p*-values were corrected for the multiple comparison testing applying the Benjamini-Hochberg false discovery rate (FDR) method (Benjamini *et al.*, 2006). Statistical significance was set p-value<0.05 after FDR correction.

RESULTS

From the total of 82 men recruited into the prospective study, the final cohort comprised of 55 participants, as certain individuals either lacked paired urine sample (N=3), failed to provide post-vasectomy samples (N=16), or had samples excluded from the analysis due to technical issues such as low DNA yield and/or poor sequencing quality (N=8). All vasectomies were uncomplicated.

Forty-six men were considered for the first comparison analysis between paired pre- and post-vasectomy semen samples; 43 post-vasectomy samples were collected 3 months after the surgery, while 3 post-vasectomy samples were taken 6 months after the intervention due to the presence of spermatozoa after 3 months of the vasectomy (N=2) or insufficient sample to evaluate the seminal parameters (N=1). For the second analysis 55 men provided paired semen and urine samples before the vasectomy. Baseline demographics, seminal parameters, and lifestyle habits are presented in **Table 1**. Regarding negative controls, 3 of them were included in the first set of analysis whereas 5 were considered for the second analysis (**Supplementary Table S1**). Reads from the negative controls were subtracted from biological samples during bioinformatics analysis.

First, we characterised the semen and midstream voided urine microbiomes separately. After applying contamination correction with microDecon and filtering out genus present in less than 30% of the samples (**Supplementary Table S2)**, the dominant phylum in semen was *Firmicutes* (45%), distantly followed by *Proteobacteria* (19%), *Actinobacteria* (16%), and *Epsilonbacteraeota* (16%). Similarly, we found *Proteobacteria* (31%) as the most abundant phylum in urine samples, closely followed by *Firmicutes* and *Bacteroides* (25% and 17%, respectively). At genus level, semen showed high abundance of *Campylobacter* (15%), *Finegoldia* (9%), and *Ezakiella* (9%)

and urine presented *Prevotella* (14%), *Acinetobacter* (7%), and *Lactobacillus* (6%) as dominant genera.

Table 1. Baseline demographics, lifestyle habits, and seminal parameters of the study participants

Participants (N = 55)	
Age (years)	40.3 ± 5.1
Body mass index* (kg/m^2)	25.9 ± 3.4
Analyses	
Pre- *vs.* Post-vasectomy	46 (83.6)
Semen *vs.* Urine	55 (100)
*Smoking**	
Never	24 (43.6)
Ex-smoker	15 (27.3)
Current smoker	15 (27.3)
*Sexual dysfunction**	
No	49 (89.1)
Occasionally	5 (9.1)
*Pre-vasectomy seminal parameters***	
Abstinence (days)	4.6 ± 6.2
Volume (ml)	2 ± 1.9
Concentration (million/ml)	65.9 ± 51.9
Progressive mobility (%)	47.1 ± 24
*Post-vasectomy seminal parameters***	
Abstinence (days)	3.01 ± 1.4
Volume (ml)	2 ± 1.6
Antibiotic oral intake in the last 3 months	
No	53 (96.4)
Yes	2 (3.6)

Data are presented as n (%) or mean ± standard deviation (SD)
*Three participants did not report their weight and height to calculate their body mass index. One participant did not answer the question regarding smoking status. One participant did not answer the questions regarding sexual dysfunction.
**Just the semen samples included in pre- *vs*. post-vasectomy comparison are considered (N=46).

Pre- vs. post-vasectomy seminal microbial analysis

Seminal microbiome α-diversity, β-diversity, and relative abundances were compared between paired pre- and post-vasectomy samples. In total, 39 unique genera were identified in the semen samples. Of these, 4 genera (*Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and *Escherichia-Shigella*, 10% of seminal genera) were exclusively characteristic to pre-vasectomy semen samples and 5 genera (*Arcanobacterium*, *Actinobaculum*, *Murdochiella*, *Howardella*, and *Fastidiosipila*, 13%) were unique to post-vasectomy samples. A total of 30 genus (77%) were common among both samples (**Figure 1A**, **Supplementary Table S3**).

Post-vasectomy semen samples had significantly higher α-diversity (observed richness Wilcoxon signed-rank *p*-value=0.011; **Figure 1B**) compared to pre-vasectomy samples. β-diversity analysis based on Bray Curtis distances indicated a significant microbial dissimilarity between seminal samples collected before and after vasectomy (PERMANOVA, R^2=0.031, *p*-value=0.004; **Figure 1C**).

Further, we performed a differential abundance analysis using ANCOM-BC to detect specific genera that could be differentially abundant in the semen microbiome of pre- and post-vasectomy samples. Ten genera showed significantly different relative abundances between pre- and post-vasectomy semen samples: *Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and *Escherichia-Shigella* showed markedly increased abundance in pre-vasectomy samples while *Arcanobacterium*, *Porphyromonas*, *Actinobaculum*, *Murdochiella*, *Howardella*, *Fastidiosipila* genera were more abundant in the post-vasectomy samples (FDR *p*-value<0.05; **Figure 2**; **Supplementary Table S4**).

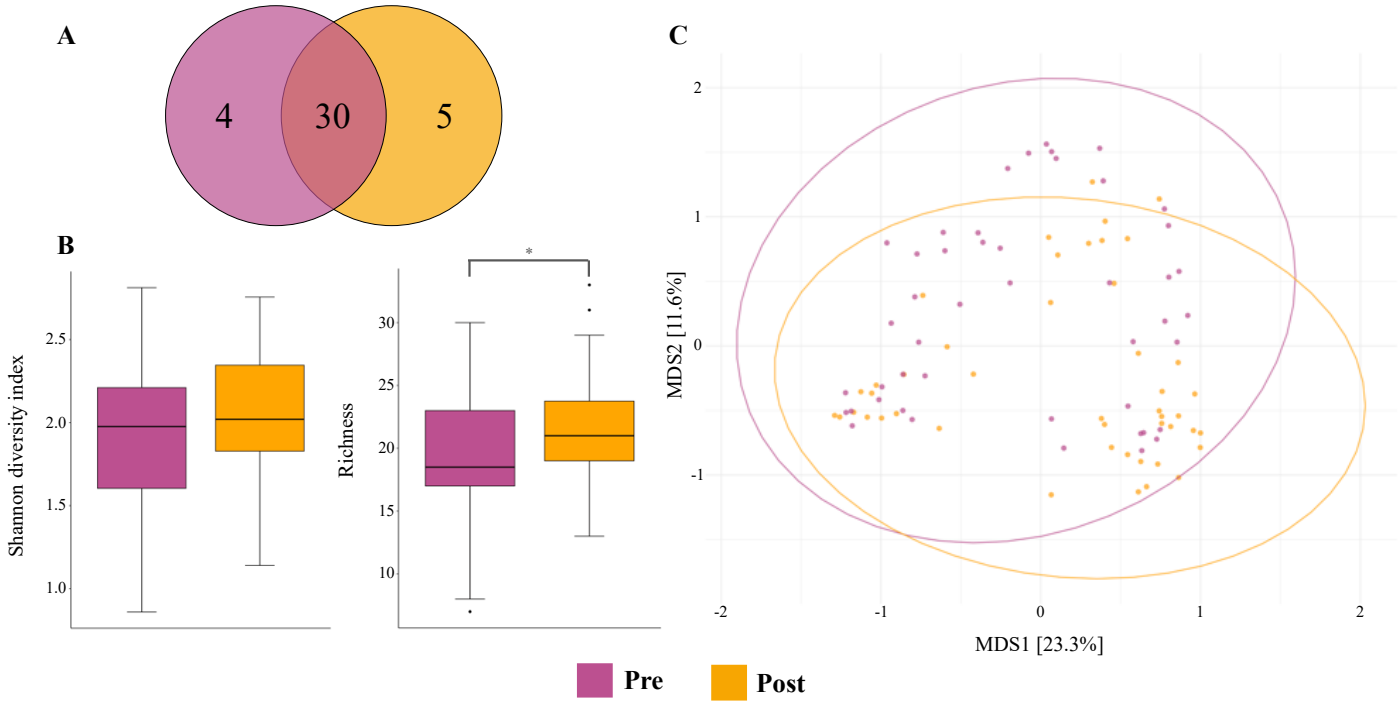

Figure 1. Comparison of bacterial genera, α- diversity, and β-diversity between paired pre- and post-vasectomy samples. A. Venn diagram showing the distribution of identified genera according to sample source. **B.** Shannon index and observed richness. Groups comparisons indicate significant difference in observed richness (Wilcoxon signed-rank, *p*-value=0.011). **C.** Nonmetric multidimensional scaling (NMDS) ordination based on the Bray Curtis distance (PERMANOVA, R^2=0.031, *p*-value=0.004).

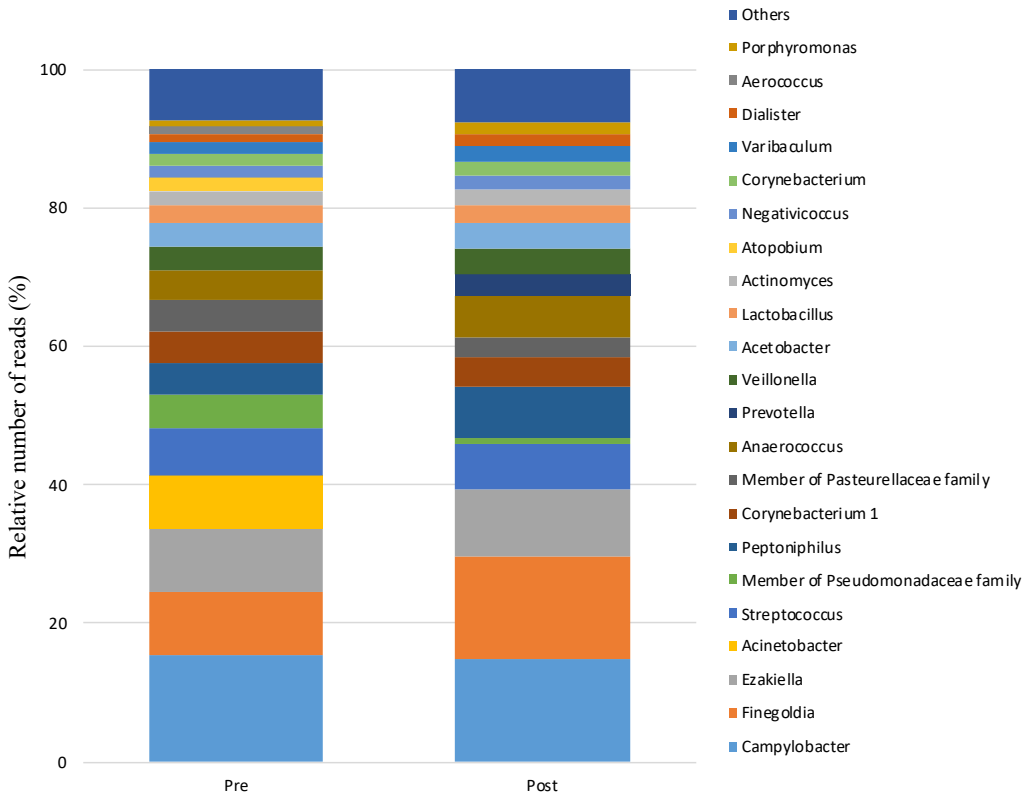

Figure 2. Relative abundance of bacterial genera in semen samples before and after the vasectomy. Genera with abundance less than 1% were grouped as "others".

Semen vs. urine microbial analysis

Seminal and urinary microbiome α-diversity, β-diversity, and relative abundances were compared between paired urine and semen samples. A total of 39 ASVs at genus level were identified. Of these, 21 genera were exclusively identified in urine samples (54% of urinal genera, **Supplementary Table S5**). None of the genera detected in this sub-analysis were unique to the semen samples. The remaining 18 genera (46%) were shared by both niches (**Figure 3A**, **Supplementary Table S5**).

Urine samples revealed significantly higher α-diversity (Shannon index and observed richness Wilcoxon signed-rank p-value<0.001; **Figure 3B**) compared to semen samples. β-diversity analysis based on Bray Curtis distances revealed discernible clustering patterns in semen and urine samples (PERMANOVA, R^2=0.117, p-value=0.001; **Figure 3C**).

Analysis of relative abundance data revealed that 31 identified genera were significantly different abundant in semen and urine samples. Among them, 21 were more abundant in urine, standing out *Prevotella* and *Escherichia-Shigella* which showed noticeably increased abundance compared to semen samples (FDR p-value<0.05, log fold change≥2.5; **Figure 4**; **Supplementary Table S6**). On the contrary, 10 genera significantly prevailed in semen samples, with particular emphasis on *Anaerococcus*, *Finegoldia*, and *Corynebacterium* (FDR p-value<0.05, fold change≥2.5; **Figure 4**; **Supplementary Table S6**).

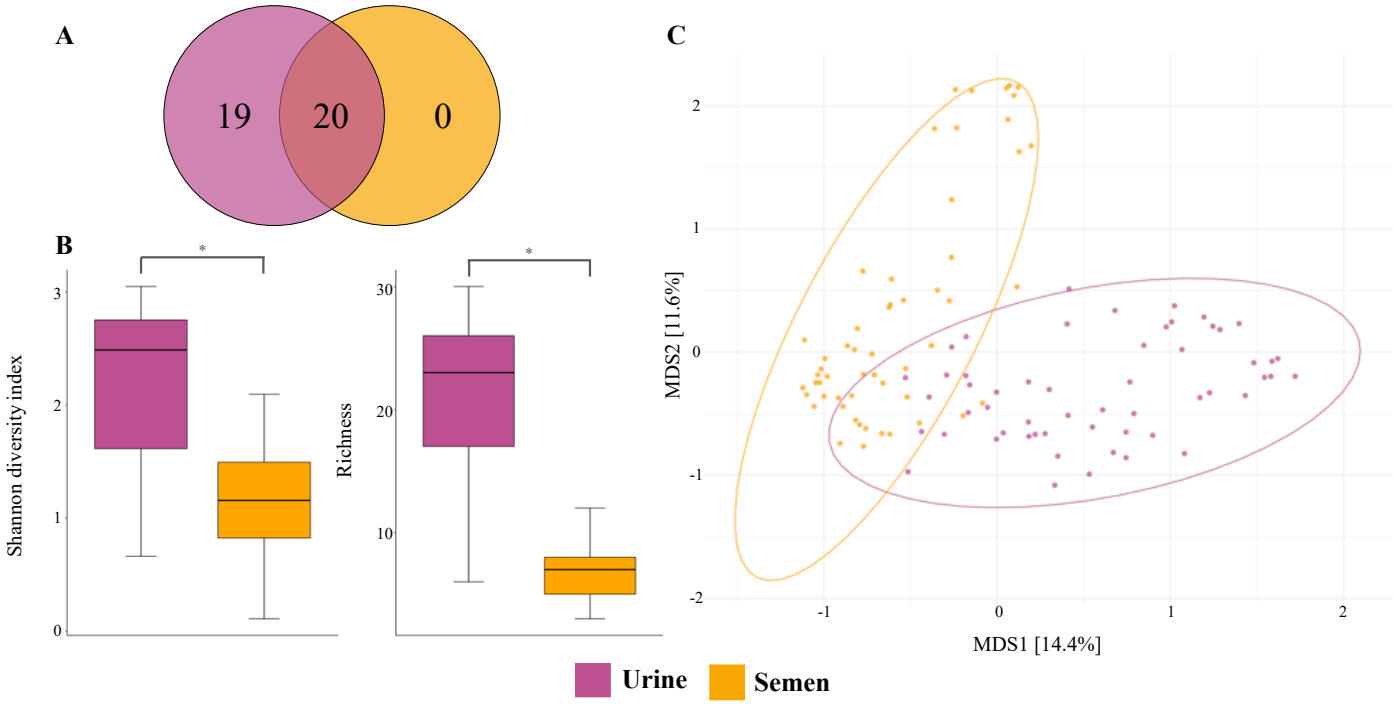

Figure 3. Comparison of bacterial genera, α- diversity, and β-diversity between paired urine and semen samples. A. Venn diagram showing the distribution of identified genera according to sample source. **B.** Shannon index and observed richness. Groups comparisons indicate significant difference in observed richness (Wilcoxon signed-rank, *p*-value<0.001). **C.** Nonmetric multidimensional scaling (NMDS) ordination based on the Bray Curtis distance (PERMANOVA, R^2=0.117, *p*-value=0.001).

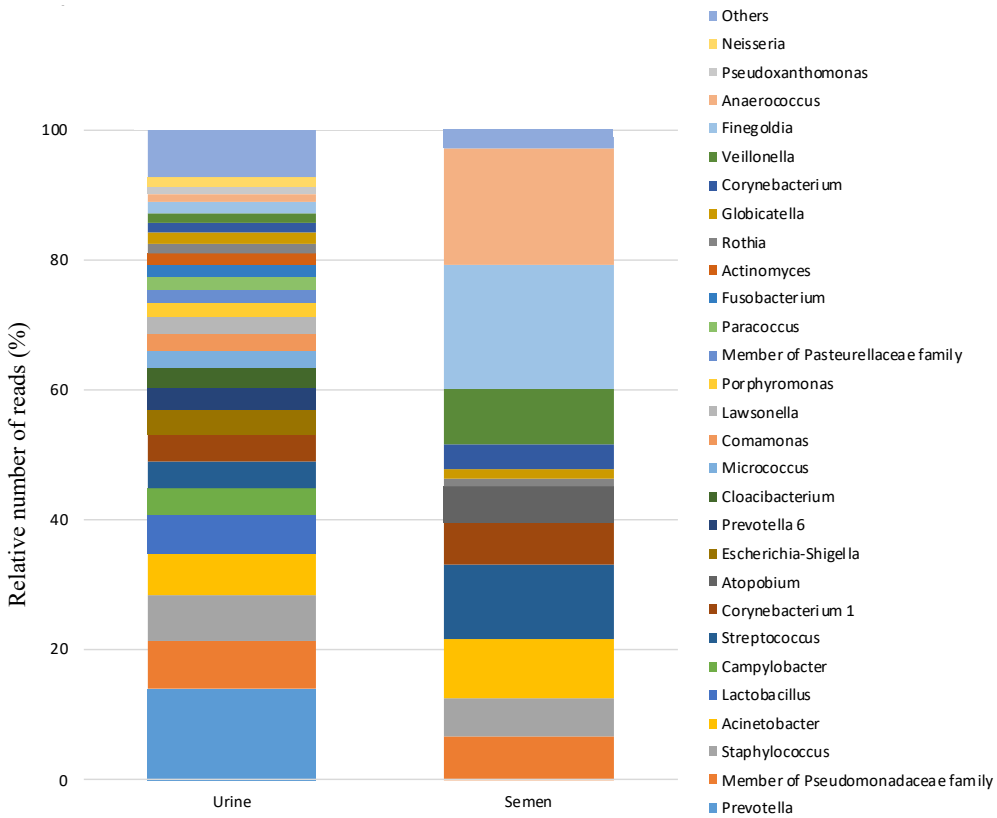

Figure 4. Relative abundance of bacterial genera in urine and semen samples. Genera with abundance less than 1% were grouped as "others".

DISCUSSION

In the current study, we described and compared the semen and urine microbiomes in paired samples from the same individuals before and after the vasectomy to better understand the origins and dynamics of the seminal microenvironment. To the best of our knowledge, this is the biggest study performed so far in the paired pre- and post-vasectomy samples. Our results indicate that vasectomy procedure influences the seminal microbial composition and that the semen shares 50% of bacterial communities with urine, altogether indicating paracrine effects of the upper reproductive tract (testis and epididymis) on seminal microenvironment.

Semen harbours its microbial communities, where we detect abundantly *Lactobacillus*, *Corynebacterium*, *Staphylococcus*, *Prevotella*, and *Finegoldia*, which is in line with previous studies (Altmäe *et al.*, 2019). When comparing the effect of vasectomy on seminal microbial composition, we found that the overall relative abundance of genera remained similar between the pre- and post-vasectomy semen samples, except for 10 genera: *Acinetobacter*, *Brevundimonas*, *Altererythrobacter*, and *Escherichia-Shigella* were decreased in post-vasectomy samples while *Arcanobacterium*, *Porphyromonas*, *Actinobaculum*, *Murdochiella*, *Howardella*, and *Fastidiosipila* were more abundant after the vasectomy. *Brevundimonas*, one of the genera we found to be significantly reduced after the vasectomy, has exhibited a reduction in vasectomised samples also in a previous study (Suarez Arbelaez *et al.*, 2023). This genus has been observed to be the most abundant in individuals exhibiting lower levels of oxidative stress, increased progressive sperm motility, and reduced levels of overall DNA fragmentation (Garcia-Segura *et al.*, 2022).

When observing the microbial richness, the vasectomy procedure had an effect of increasing the α-diversity among the seminal samples, with significant differences in

genera richness. In line with our finding, a pioneering study found that only two of the pre-vasectomy samples, but all five of the post-vasectomy samples, tested positive for bacteria (Kiessling *et al.*, 2008). While other authors in a limited sample size have detected the contrary, vasectomy led to a decrease in α-diversity in paired and unpaired semen samples (Suarez Arbelaez *et al.*, 2023). There seems to be consistency in affecting the composition and abundance of the seminal microbiome. This suggests that the upstream anatomic locations such as testis and epididymis have their unique microbiome and that the paracrine contribution of these sites can influence to the seminal microbial composition. Indeed, a testicular microbiome has been described, albeit as low biomass site (Alfano *et al.*, 2018; Molina *et al.*, 2021a). Further, supporting our results earlier studies on the semen microbiome have demonstrated a correlation between dysbiosis and an increase in bacterial richness (Kiessling *et al.*, 2008; Altmäe *et al.*, 2019; Contreras *et al.*, 2023; Zuber *et al.*, 2023). In our study, an increase in the bacterial richness in the post-vasectomy samples could be explained by the absence of testicular and epididymal influence on semen microbiota after the vasectomy. The removal of these contributions may allow for other bacterial sources, possibly from the urinary tract or external genitalia, to become more prominent in the semen, thereby increasing the diversity and richness of the microbiome. Further, this rise in bacterial richness may be also linked to the epididymis-unique defensins (Yamaguchi *et al.*, 2002; Yenugu *et al.*, 2004). Defensins are a group of antimicrobial proteins recognised as vital in response to pathogens. Humans are known to produce a reasonably large quantity of these defensins in their epididymis, including certain types that are exclusive to this organ (Kiessling *et al.*, 2008). In light of our study results, one could conjecture that these epididymal defensins might act as a protective shield against bacterial infections in downstream tissues. Thus, further studies are needed to identify the specific bacteria that are lost post-vasectomy and to

understand the exact biological mechanisms they may have in a short and long term on male health.

Our study findings exposed a statistically significant difference in α- diversity between semen and urine samples, with urine exhibiting higher diversity. Previous studies have obtained contrary results, detecting higher α-diversity in semen (Lundy *et al.*, 2021) or no differences in microbial α-diversity between semen and urine (Cao *et al.*, 2023). The difference could arise from small sample size analysed in previous studies, and additionally Cao *et al.* study collected semen samples first, followed by urine samples, our study followed the reverse order, collecting urine before the semen.

In addition to α-diversity, also the other diversity measure, β-diversity, significantly changed after vasectomy, supporting that semen microbial communities fluctuate after male sterilisation. In line, the only study where diversity between non- and vasectomised samples has been analysed so far, the bacterial composition of the samples did show a tendency for distinct clustering between the two groups, nevertheless due to the small sample size (i.e., 16 individuals) the result was not statistically significant (Suarez Arbelaez *et al.*, 2023).

Our analysis of the microbiome profiles between the paired semen and urine samples revealed that the β-diversity analysis resulted in discernible clustering patterns meaning that the two types of samples have unique and distinct sets of bacterial genera, which is also observed by previous studies (Lundy *et al.*, 2021; Cao *et al.*, 2023).

When we compared the microbial composition between the semen and urine to disentangle further the seminal microbial origin, semen displayed higher *Anaerococcus, Finegoldia, and Corynebacterium* abundances and reduced *Prevotella and Escherichia-Shigella* among others. All these genera have been previously described in both niches

(Cao et al., 2023; Lundy et al., 2021). Interestingly, *Prevotella* has been broadly linked to reduced parameters of semen quality (Nguyen *et al.*, 2014; Weng *et al.*, 2014; Baud *et al.*, 2019; Farahani *et al.*, 2020; Cao *et al.*, 2023) and its abundance has shown significant differences between urine and semen. In our study and others, it has been found to be more abundant in urine (Lundy *et al.*, 2021), while contrasting findings from other authors have reported lower abundance in urine (Cao *et al.*, 2023). Likewise, we observed a similar pattern for other bacterial genera, which were more abundant in semen, occasionally coinciding with the literature, as is the case for *Finegoldia* (Cao *et al.*, 2023), *Lactobacillus* (Lundy *et al.*, 2021), and *Enterococcus* (Lundy *et al.*, 2021). However, disparities arise when considering *Anaerococcus, Veillonella*, *Corynebacterium*, and *Streptococcus*, as our findings indicate greater abundance in semen, in contrast to other studies (Lundy *et al.*, 2021; Cao *et al.*, 2023). Also, for bacteria abundant in the urine in our study, such as *Prevotella, Lactobacillus, Escherichia-Shigella*, and *Porphyromonas* contradicting results in other studies have been obtained (Lundy *et al.*, 2021; Cao *et al.*, 2023). However, we did observe a slightly higher abundance of *Bifidobacterium* in urine, consistent with another study (Cao *et al.*, 2023). These contradicting results between studies could arise from different sample size and study design, protocol used and analysis methods (Molina *et al.*, 2021b). Indeed, the biggest discordancy between our study findings was found with the study by Cao *et al*., where the semen samples were collected first, followed by urine samples (while in our study the order was reverse). Further, one plausible explanation for the shared presence of these genera in both urine and semen could be the anatomical proximity of the urethra (through which urine passes) and the vas deferens (which transports sperm). Cross-contamination could occur during urination or ejaculation due to their common exit pathway from the body. It is also conceivable that urethral colonisation by some of these genera could subsequently influence the seminal

microbiome. Another possible explanation could be related to biofilm formation. Many of these genera are known for their biofilm-forming capabilities (Davey and O'toole, 2000; Hall-Stoodley *et al.*, 2004; Kolenbrander *et al.*, 2006; Brook, 2007; Turroni *et al.*, 2014; Souza *et al.*, 2015), which could allow them to persist in the genitourinary tract, colonise both the urinary and reproductive systems, and possibly influence the microbiome composition of both niches. Nonetheless, the mechanisms behind the differential abundance in semen and urine, as well as its potential effects on sperm quality, require further investigation.

The strength of our study is the increased sample size and that the same individuals were assessed before and after the vasectomy and paired comparisons were made for semen and urine microbiomes which eases the impact of population and lifestyle factors on the microbial composition outcome while providing a more comprehensive understanding of the specific microbiome changes. In fact, a previous study where paired and unpaired seminal samples before and after the vasectomy were analysed, the paired samples identified significantly less bacterial species between study groups than the unpaired samples (Suarez Arbelaez *et al.*, 2023). Also, contamination in microbiome analysis was stringently controlled including negative and positive controls together with *in silico* decontamination methods. Nevertheless, the study has limitations that should be mentioned. Initially, it is worth noting that mid-stream urination and masturbation involve the urethra, which harbours the urethral microbiome. Although catheterisation and seminal vesicle aspiration are more suitable collection methods to elucidate seminal microbial origin, it is improbable that volunteers would accept. Another limitation was obtaining sufficient DNA yield from semen samples, which presents a challenge during sequencing. This difficulty in obtaining an adequate amount of bacterial DNA

complicates the sequencing process and requires a larger initial sample size to account for potential sample dropouts.

CONCLUSION

In the exploration of the seminal microbiome origin through the impact of vasectomy and comparison with the urinary system we analyse paired seminal and urinal pre- and post-vasectomy samples within the largest cohort to date. Our findings reveal considerable differences in both α- and β-diversity indices when comparing pre- and post-vasectomy semen samples as well as between urine and semen samples. Intriguingly, we have also pinpointed several bacterial genera that show significant variations in abundance across the different niches examined. Altogether, our study underscores the intricate relationships between anatomically close but functionally distinct niches within the male reproductive and urinary systems. The differential microbial community structures and compositions might be associated with different physiological states and could potentially influence the health outcomes. Our study findings provide new insight into the origin of seminal microbes, indicating that some accompanying bacteria could already originate from the testicular and urinary environment.

By elucidating the origins of the seminal microbiome, this work will provide crucial insights into the factors influencing male reproductive health and demonstrate that the vasectomy procedure might have long lasting effects on male health via modulation of seminal microenvironment. A comprehensive understanding of the seminal microbiome's origin and its impact on male fertility will pave the way for novel diagnostic approaches, therapeutic interventions, and strategies for promoting reproductive health.

SUPPLEMENTARY MATERIAL

In addition, the supplementary files can be downloaded in this link: https://osf.io/z8sha/?view_only=77019cb7048f454da8b98d7e19cad96f

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Study III: The seminovaginal microbiome: it takes two to tango

ABSTRACT

Infertility, adverse pregnancy outcomes, and genital infections are prevalent, affecting millions of couples worldwide. The seminal and vaginal microbiome appear to play an important role in the physiology and pathophysiology of the male and female reproductive tracts. Despite of the shared body fluids containing thousands of microbes during unprotected sexual activity, they have traditionally been studied separately, with greater emphasis placed on the vaginal microbiota. Consequently, the concept of the "seminovaginal microbiota" emerges to address both microbial niches equally and to provide holistic explanations and solutions to these reproductive issues. This systematic review discusses the status of the complementary microbiome, encompassing its diversity and composition, and how it is linked to the health and disease of the couple, the success of assisted reproductive techniques and pregnancy.

INTRODUCTION

The human body is colonised with more bacteria than human cells in the body (NIH HMP Working Group *et al.*, 2009; Proctor *et al.*, 2019). The microorganisms that colonise our body are known as our microbiota which, in addition to bacteria, includes viruses, fungi, yeasts, archaea, and protozoa (Cho and Blaser, 2012; Ursell *et al.*, 2012). The genetic content of these microorganisms and the surrounding environmental conditions are termed as the microbiome (Marchesi and Ravel, 2015). Each individual has a unique mix of microbes, presumably as a result of genetic, epigenetic, and environmental factors that regulate bacterial colonisation and its stability (Peery *et al.*, 2021).

The fact that each human being is populated by a different combination of microorganisms makes us more or less susceptible to certain diseases (Li *et al.*, 2020). Impaired reproduction, adverse pregnancy outcomes, and genital infections are challenges that impact couples worldwide (Tsonis *et al.*, 2021). In fact, ~15% of male infertility cases are due to infection and inflammation of the urogenital tract induced by microbiological factors, while sexually transmitted infections (STIs) remain the primary cause of female infertility (Dohle, 2003; Gimenes *et al.*, 2014). The microbiome present in the semen and vagina have been found to play a significant role in the functioning of the male and female reproductive systems (Mändar, 2013; Altmäe *et al.*, 2019; Koedooder *et al.*, 2019; Molina *et al.*, 2020, 2021; Okwelogu *et al.*, 2021; Sola-Leyva *et al.*, 2021). Despite of the shared body fluids containing thousands of microbes during unprotected sexual activity, the male and female urogenital microbial niches have traditionally been studied separately, with a stronger focus on the vaginal microbiome (Amato *et al.*, 2020). Indeed, a minimal number of studies have focussed on the interacting microbiome analysis of both partners' (Mändar *et al.*, 2015; Zozaya *et al.*, 2016; Plummer *et al.*, 2018, 2021; Amato *et al.*, 2020; Campisciano *et al.*, 2020; Mehta

et al., 2020a, 2020b, 2022b, 2022a; Manzoor *et al.*, 2021; Okwelogu *et al.*, 2021; Iniesta *et al.*, 2022; Baud *et al.*, 2023; Koort *et al.*, 2023), mainly due to the study question and the complexity involved in simultaneous collection of samples from both individuals. Thus, the concept of the "seminovaginal microbiota" that was proposed in 2015 (Mändar *et al.*, 2015), has not gained much attention and its short- and long-term potential in human urogenital health and reproduction awaits to be fully established and understood.

The seminovaginal microbiota comprises of all the microorganisms from seminal and vaginal ecosystems that are transferred and shared between the partners during unprotected sexual intercourse, influencing each other and impacting reproductive health and functions (Mändar *et al.*, 2015). The broader concept of the shared reproductive microbiome encompasses microbes residing in areas or bodily fluids that interact with couple's gametes or reproductive organs during sex (Rowe *et al.*, 2020). This can include microbes from other body regions like the oral or perianal areas (Verstraelen *et al.*, 2010; Carda-Diéguez *et al.*, 2019; Williams and Gibson, 2019), reflecting different sexual activities and partners. Further, this bidirectional exchange can influence the microbial make-up of either partner or potentially both (Koort *et al.*, 2023). Indeed, studies are demonstrating that bacteria are shared among partners and that they influence the species composition of the couple's reproductive tract (Mändar *et al.*, 2015; Zozaya *et al.*, 2016; Plummer *et al.*, 2018, 2021; Amato *et al.*, 2020; Campisciano *et al.*, 2020; Mehta *et al.*, 2020a, 2022b; Manzoor *et al.*, 2021; Okwelogu *et al.*, 2021; Iniesta *et al.*, 2022; Baud *et al.*, 2023; Koort *et al.*, 2023). Further a hypothesis of the vaginal microbiome directly affecting male genital tract health leading to chronic infection of prostate has been proposed (Reece, 2017). Therefore, these microbial communities may have far-reaching implications for individual and the couple, which is up to date understudied and weakly determined. With this systematic review we aim to provide the current knowledge of

seminovaginal microbiome studies, to assess the shared microbes within couple, and to determine the potential impact of the shared microbiomes on couple's health.

MATERIAL & METHODS

The search strategy was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines (**Supplementary Table S1)** (Page *et al.*, 2021). The review protocol has been registered in the International Prospective Register of Systematic Reviews (PROSPERO, CRD42022323201).

Data source and search strategy

A systematic search of the literature in PubMed, Web of Science, and Scopus was independently conducted up to June 2023 by two researchers. The strategy performed for literature search combined keywords and medical subject heading (MeSH). The search was focussed on male and female reproductive niches, microbiota/microbiome, and human reproduction related words. Detailed search query is reported in **Supplementary Table S2**.

Study selection

The study population consisted of couples at their reproductive age. All types of studies describing the microbial composition of genital tract in female (i.e., vagina) and male (i.e., semen, penile skin) genital tracts of couples via the NGS were included. The exclusion criteria were conference abstracts, letters to editors, study protocols, editorials/opinions, case reports, non-full text availability, review articles, or studies

assessing the microbial composition in one of the partners' parts and studies written in any language other than English or Spanish. Time (from 2007 to the present) and human-specie filters were applied.

Study selection was completed independently by two investigators and discrepancies were discussed and solved by involving a third independent researcher. To start, resulting articles from the systematic search were screened by the title and abstract whereafter irrelevant articles were removed. Afterwards, full-text screening of the remaining articles was conducted.

Following systematic search and study selection, additional records were hand-searched using the snowballing method to identify other potentially eligible studies. This method helps to ensure that all relevant literature has been identified as extra studies were retrieved based on the reference lists of review articles and previous selected studies.

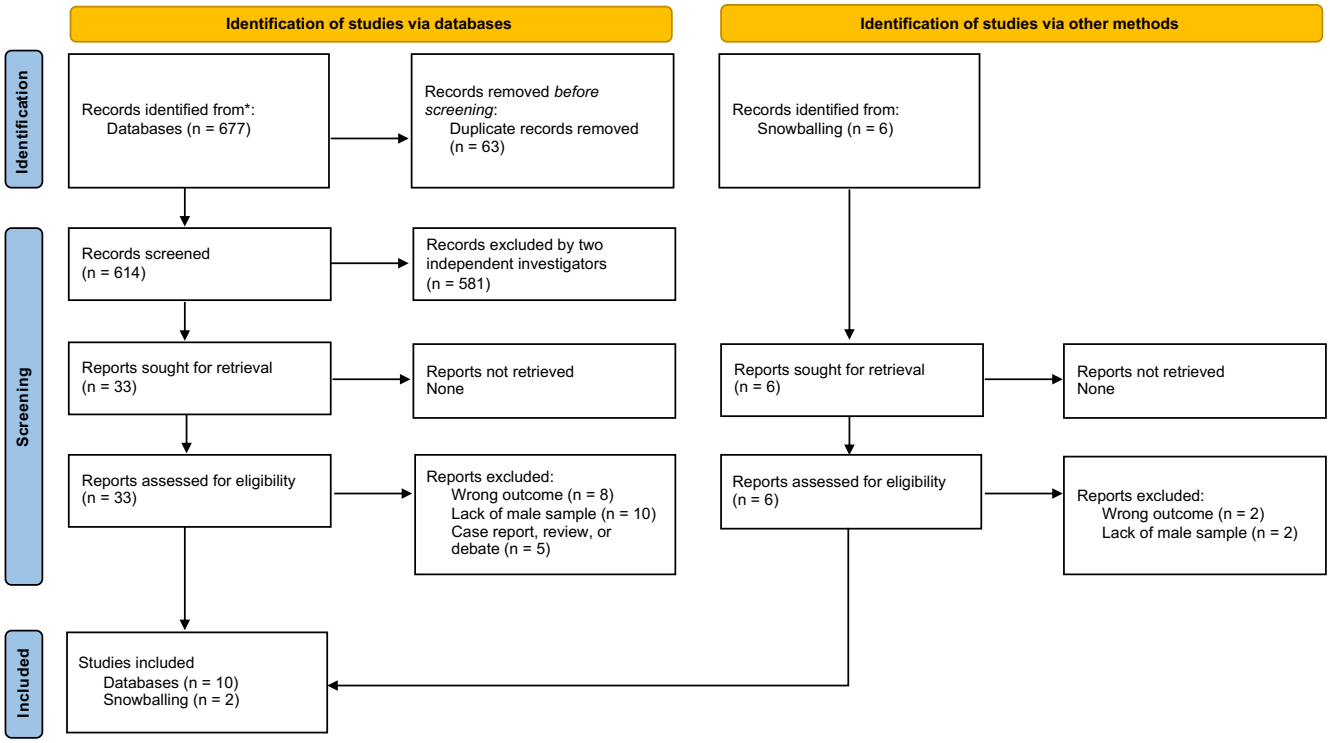
Data extraction and synthesis

The primary outcome of this review was to identify the shared microbiome profiles within the couple. Data from selected articles were manually extracted by two investigators. For every eligible study during full-text screening the following information was gathered: 1) reference information; 2) study aim; 3) study design; 4) study population (number of participants, condition, age, country/ethnicity, possible treatment); 5) sampling (body niche, collection procedure, follow-up); 6) top identified taxa in each of the individuals of the couple and the shared ones; and 7) main study conclusions.

RESULTS AND DISCUSSION

Identification and selection of articles

The PRISMA flowchart of the search strategy, identification, and selection process is depicted in **Figure 1**. Initial searches identified a total of 677 articles, including 63 duplicates which were removed. The remaining 614 articles were screened for title and abstract whereafter 581 records were excluded, and 33 articles were selected for the full-text evaluation. Twenty-three articles were excluded based on inclusion and exclusion criteria: wrong outcome (i.e., not microbial composition from sequencing methods, N=8), not inclusion of male samples (N=10), and case report, review, or debate (N=5). Ten articles met the inclusion criteria and were selected via the systematic search. Additional records were included using snowballing method (N=2). Eventually, 12 studies were included (**Table 1**).



*Records identified from PubMed, Web of Science, and Scopus databases.

Figure 1. PRISMA 2020 flow diagram for systematic review. Study identification, screening, and eligibility.

Seminovaginal microbiome in different-sex partners

Semen serves as a canal for microbial exchange during sexual intercourse. Sexually experienced men were shown to display greater bacterial diversity and concentration than men at same age who have never had sex (Mändar *et al.*, 2018). Seminal neutral to slightly alkaline pH, around 7.5, can impact the acidic environment of the vagina during unprotected sex, potentially leading to shifts in microbial composition, including increased bacterial vaginosis (BV)-related bacteria (Fox *et al.*, 1973). However, changes in the vaginal microbiota are not solely pH-dependent; the microbes within the semen itself also contribute to alterations in the vaginal microbiome (Hou *et al.*, 2013; Mändar *et al.*, 2015, 2018). For instance, a considerable correlation between the presence of sperm in vaginal samples and the Nugent score (i.e., a measure of BV) has been demonstrated (Jespers *et al.*, 2014). Further, several genera found in semen have been negatively associated with the vaginal health (Borovkova *et al.*, 2011; Mändar *et al.*, 2015; Onderdonk *et al.*, 2016; Baud *et al.*, 2023), including *Prevotella* that associates with BV (Onderdonk *et al.*, 2016). In the context of sexual behaviour, factors such as the number of sex partners, condom use, and the time since the last sexual intercourse, have been associated with the composition of the male reproductive microbiome (Mändar *et al.*, 2017; Mehta *et al.*, 2022b).

The make-up of the seminal microbiome varies greatly from individual to individual, especially regarding the composition and relative abundance of different microorganisms (Hou *et al.*, 2013; Weng *et al.*, 2014; Manzoor *et al.*, 2021). The main genera found in semen include *Lactobacillus*, *Finegoldia*, *Prevotella*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus*, among others (Altmäe *et al.*, 2019; Contreras *et al.*, 2023; Zuber *et al.*, 2023). The seminal microbiome is not static, as it can undergo alterations based on a man's health conditions and factors like lifestyle habits (such as hygiene and diet), age,

ethnicity, existing, and the use of antibiotics and probiotics (Altmäe *et al.*, 2019). Nonetheless, long-term studies that periodically sample the same individuals over time are needed to fully understand the stability and fluctuations of the seminal microbiome.

In healthy women of reproductive age, their vaginal environment is typically characterised by a microbiome with limited diversity, mainly composed of bacteria from *Lactobacillus* genus (Ravel *et al.*, 2011). *Lactobacillus* spp. contribute to a low vaginal pH, suppressing harmful bacterial growth (Ma et al., 2012; Mitchell et al., 2015). Indeed, a healthy lactobacilli-dominant vaginal microbiome is associated with better reproductive outcomes (Haahr *et al.*, 2016). The vaginal microbial composition has a dynamically changing landscape which can vary significantly daily and weekly (Song *et al.*, 2020), although it can also maintain stability over several months (Gajer *et al.*, 2012). Notably, these fluctuations appear to be influenced by a multitude of factors including the menstrual cycle, sexual activity, hormonal contraceptive use, diet, exercise, and antibiotic/probiotic use (Eschenbach *et al.*, 2000; Kaminska and Gajecka, 2017).

Associations between the vaginal and seminal microbiomes

The seminal microbiome is more diverse, albeit with a lower bacterial concentration than the vaginal microbiome (Mändar *et al.*, 2015; Wang *et al.*, 2020; Manzoor *et al.*, 2021; Iniesta *et al.*, 2022; Baud *et al.*, 2023; Koort *et al.*, 2023). This implies for a complex and enriched bacterial community in semen that could interact with the more concentrated but less diverse vaginal microbiota during sexual intercourse.

Seminovaginal microbiome has been mostly studied among couples with infertility who come to the clinic for infertility treatment. The first NGS study comparing pre- and post-coital vaginal and semen samples from 23 couples with infertility reported changes in

both the seminal and vaginal microbiomes following sexual intercourse (Mändar *et al.*, 2015). Both partners shared many bacterial genera, including *Veillonella*, *Porphyromonas*, *Atopobium*, *Streptococcus*, and *Lactobacillus* following the intercourse. Women, however, had more bacteria from the *Firmicutes* phylum, while men were dominated by bacteria from the *Bacteroidetes* and *Proteobacteria* phyla (Mändar *et al.*, 2015). These latter phyla revealed a strong correlation with inflammation in the male genital tract, and the abundance of *Proteobacteria* was particularly associated with men suffering from leukocytospermia (Mändar *et al.*, 2015). It is worth mentioning that *Proteobacteria* phylum comprises a large number of human pathogens (Manzoor *et al.*, 2021). In another study, leukocytospermia was linked with a high level of bacteria in the semen and sperm damage due to the formation of ROS (Fraczek *et al.*, 2007). Additionally, leukocytospermia has been significantly correlated with the presence of *Gardnerella vaginalis* in the vaginal microbiota and adverse pregnancy events (Kjaergaard *et al.*, 1997; Wittemer *et al.*, 2004). This suggests that the presence of *Proteobacteria* in sperm might predispose women to have *G. vaginalis* in their microbiota and to a state of temporary BV. However, *Protebacteria* phylum itself has been over-represented in women with infertility compared to fertile women and linked to BV and adverse pregnancy outcomes (Manzoor *et al.*, 2021). Further, the genus *Gardnerella* is frequently found in semen (Altmäe *et al.*, 2019), suggesting that some of the increase in *G. vaginalis* after unprotected sex may be due to transmission from the seminal microbiota (Vodstrcil *et al.*, 2017).

Sexually active young men exhibited a higher prevalence of typical vaginal microbiota species in the semen such as *Lactobacillus crispatus*, *L. iners*, *G. vaginalis*, and *Atopobium. vaginae*, while sexually less active older men harboured more bacteria in the semen from *Pseudomonas*, *Gillisia*, *Flavobacterium*, and *Acidovorax* genera (Mändar *et*

al., 2017), which refers to microbial differences due to sexual activity and/or age. Interestingly, the onset age of sexual activity and the frequency of sexual encounters have been shown to affect the seminal microbial composition (Vodstrcil *et al.*, 2017; Mändar *et al.*, 2018).

Impact on seminal parameters. Evidence from recent studies shows that couples having unprotected sexual intercourse share certain bacterial genera that can impact seminal parameters (Altmäe et al., 2019). In semen, *Lactobacillus* relative abundance is significantly lower and their roles are not well-defined (Koort *et al.*, 2023). Recently, increased abundance of *Lactobacillus* in the seminal microbiome was correlated with improved sperm motility and concentration and with normal morphology, probably because it prevents lipid peroxidation (Moretti *et al.*, 2009; Weng *et al.*, 2014; Baud *et al.*, 2019). Accordingly, *Lactobacillus* spp. have garnered considerable attention due to their probiotic potential for semen quality maintenance and how probiotic interventions with *Lactobacillus* strains have influenced the seminal microbiome (Wang *et al.*, 2022). Another vaginal bacterium found to negatively affect sperm health and associate with infertility in men is *G. vaginalis* which has also been linked with BV when it outnumbers *Lactobacillus* spp. in women (Mändar et al., 2015).

Interestingly, the adhesion of *E. coli* to sperm has been demonstrated and has been correlated to diminished embryo quality by promoting spermatozoa agglutination via their plasma membranes and their subsequent destruction by inducing cell apoptosis (Moretti *et al.*, 2009; Kala *et al.*, 2011; Fraczek *et al.*, 2012). Further, some vaginal strains of *E. coli* are implicated in causing urinary tract infections and have been associated with sperm dysfunction and male infertility (Cottell et al., 2013; Sanocka-Maciejewska et al., 2005).

Also, the impact of *Klebsiella pneumoniae* and *S. agalactiae*, common opportunistic bacteria in the vagina, on sperm parameters has been studied *in vitro*, as well as their capacity to interact with and be transported by human sperm (Zuleta-González *et al.*, 2019). The findings revealed that the presence of *K. pneumoniae* adversely impacted sperm motility, specifically the progressive motility that is crucial for successful fertilisation. Additionally, the bioactive substances released by this bacterial specie influenced sperm health, increasing the number of necrotic sperm cells. Similarly, the soluble factors of *S. agalactiae* led to an increase in lipid peroxidation in the sperm membrane, a process that can damage cell structures and potentially impair sperm function. These authors observed a robust interaction between sperm and these bacteria and concluded that human sperm might act as vehicles for these bacteria, facilitating their spread within the female reproductive tract (Zuleta-González *et al.*, 2019).

ART outcomes. Due to the direct clinical interest, studies have started to elucidate the link between the seminovaginal microbiome and ART outcomes. Correlations between specific bacterial proportions and positive IVF outcomes have been described (Okwelogu *et al.*, 2021). Higher concentrations of *Alphaproteobacteria* (class), *Gammaproteobacteria* (class), and *Corynebacterium* in semen microbiome has been associated with lower embryo quality, while a higher abundance of *Enterobacteriaceae* (family) and *Lactobacillus* was correlated with better embryo quality (Štšepetova *et al.*, 2020). Further, in semen samples, the increased mean proportions of *L. jensenii* and *L. iners* and decreased proportions of *Proteobacteria* and Gram-negative anaerobes have been associated with IVF success (Okwelogu *et al.*, 2021).

Table 1. Systematic search results and comprehensive overview of data extracted from microbiome studies in different-sex reproductive-age couples using 16S rRNA gene sequencing

Reference information	Study aim	Study design	Population (N, diagnosis, ethnicity, age [y], treatment)	Sampling	Top identified taxa	Main conclusions
(Mändar et al., 2015)	To compare seminal and vaginal microbiomes in couples and to assess the influence of sexual intercourse on vaginal microbiome	Longitudinal	23 couples with infertility (Estonia) Men 32.2 (24-43) y Women 29.9 (21-39) y Tx No	Semen aliquot Vaginal swab F/U <24 hours	Men *Varibaculum*, *Flavobacterium*, *Prevotella*, *Porphyromonas*, *Dysgonomonas*, *Atopobium*, *Corynebacterium* Women *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Veillonella*, *Pseudomonas*, *Atopobium* Shared 85% of all detected phylotypes *Lactobacillus*, *Veillonella*, *Streptococcus*, *Porphyromonas*, *Atopobium*	Seminal communities were significantly more diverse, but with lower total bacterial concentrations than those of the vagina Significant decrease in the relative abundance of *L. crispatus* after intercourse and high concordance between semen and vaginal samples
(Zozaya et al., 2016)	To examine the diversity, community composition, prevalence, and relative abundance of genital bacteria in monogamous couples	Cross-sectional	65 couples where the female has BV (Africa-American) Men 30 ± 8.4 y Women 27.3 ± 6.6 y Tx No	Penile swab Vaginal swab F/U No	Men *Corynebacterium,* *Peptoniphilus*, *Anaerococcus*, *Staphylococcus*, *Finegoldia*, *L. iners*, *Streptococcus*, *Gardnerella*	Diversity of BV couples was higher in penis and vagina compared to non-BV couples The penile skin communities of BV-males were significantly more similar to

			31 couples without BV (African-American) Men 32.2 ± 12 y Women 28.4 ± 7.7 y		Women *L. iners*, *Gardnerella*, *L. crispatus*, *Megasphaera*, *Enterobacter* Shared most OTUs in vaginal and penile samples of BV couples showed a strong positive correlation, while correlations among non-BV couples were strikingly lower *Peptoniphilus*, *Gardnerella*, *Lactobacillus*, *Barnesiella*, *Prevotella*, *Megasphaera*, *Dialister*	the vaginal communities of their sexual partner
(Plummer et al., 2018)	To investigate the impact of dual-partner BV treatment on the vaginal and penile microbiome	Longitudinal (pilot trial)	21 couples where the female has BV (mainly Australia) Men 33.1 ± 9.1 y Tx oral metronidazole (400mg twice daily for 7 days) and clindamycin applied topically to penile skin (2% cream twice daily for 7 days) Women 28.6 ± 6.4 y	Penile swab Vaginal swab F/U 4 weeks	Men *Corynebacterium*, *Finegoldia*, *Peptoniphilus*, *Prevotella*, *Staphylococcus*, Women *Gardnerella, L. iners*, *Prevotella*, *L. crispatus*, *Staphylococcus*, *Escherichia*/*Shigella* Shared *Dialister*, *Prevotella*, *Staphylococcus*	Correlations between prevalent taxa in vagina and penile microbiome The vaginal microbiome was not more similar to the cutaneous penile microbiota of their sexual partner, when compared to non-partner males Re-emergence of BV-associated bacteria in the penile microbiome post-treatment was common

			Tx oral metronidazole (400mg twice daily for 7 days) or intravaginal clindamycin (2% applicator vaginally for 7 nights)			
(Amato et al., 2020)	To characterize the vaginal and seminal microbiome in couples with idiopathic infertility undergoing their first IUI and to correlate it with the clinical pregnancy rate after IUI	Observational prospective	23 couples with idiopathic infertility (Italy) Men 34 ± 4 y Women 33 ± 3 y Tx No	Semen aliquot Vaginal swab F/U No	Men *Tissierellaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Prevotellaceae*, *Corynebacteriaceae* Woman *Lactobacillaceae*, *Pseudomonadaceae*, *Moraxellaceae*, *Pasteurellaceae*, *Enterobacteriaceae*, *Campylobacteraceae*, *Tissierellaceae*, *Veillonellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Streptococcaceae* Shared *Tissierellaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Prevotellaceae*, *Corynebacteriaceae*	Changes in the composition of the vaginal microbiome (but not the seminal microbiome) were associated with successful outcomes of IUI Potential role of *L. crispatus* in promoting a favourable environment for pregnancy
(Mehta et al., 2020)	To identify factors associated with clinical BV among	Longitudinal	252 heterosexual couples (Kenya) Men median 27 y	Penile swab Cervicovaginal lavage	Men *Corynebacterium*, *Anaerococcus*, *Finegoldia*, *Sneathia*	Penile bacteria were associated with increased odds of Nugent BV among

	women with nonoptimal vaginal microbiome/molecular BV		Women median 22 y Tx No	F/U 12 months	*sanguinegens*, *Streptococcus* Women *Prevotella*, *L. iners*, *Atopobium vaginae*, *Dialister*, *Megasphaera*, *Prevotella* Shared *Finegoldia*, *Streptococcus*, *S. sanguinegens*. *Dialister*, *Prevotella*	women with nonoptimal CST Partner circumcision associated with a reduced risk of BV in female partners
(Plummer et al., 2021)	To assess the impact of concurrent BV partner treatment on genitourinary sites over a 12-week period	Prospective open-label pilot	34 couples where the female has BV (mainly Australia) Men 31 (27–37) y Tx oral metronidazole (400mg twice daily for 7 days) and clindamycin applied topically to penile skin (2% cream twice daily for 7 days) Women 30 (27–34) y Tx oral metronidazole (400mg twice daily for 7 days) or intravaginal clindamycin (2% applicator vaginally for 7 nights)	Penile swab Vaginal lavage F/U 12 weeks	Men *Staphylococcus*, *Finegoldia*, *Prevotella*, *Corynebacterium*, *L. iners*, *Peptoniphilus* Woman *L. iners*, *Gardnerella*, *Prevotella*, *Sneathia*, *Peptoniphilus* Shared *Candidatus Lachnocurva vaginae* (BVAB-1), *L. crispatus, L. gasseri*, *Corynebacterium*, *Finegoldia*, *Aerococcus*, *Prevotella*	Immediately post-treatment, concurrent partner treatment significantly reduced the abundance of BV-associated bacteria and altered the overall vaginal and seminal microbiome composition A significant positive correlation of taxa between sexual partners longitudinally, being less sustained in male microbiome

(Manzoor et al., 2021)	To characterise the microbiome associated with fertile and infertile couples	Cross-sectional	23 couples with infertility (Pakistan) Tx No Men 33.97 ± 6.14 y Women 28.25 ± 5.47 y 22 fertile couples (Pakistan)	Genital swabs F/U No	Men *Corynebacterium*, *Staphylococcus*, *Lactobacillus*, *Anaerococcus*, *Finegoldia*, *Prevotella* Woman *Lactobacillus*, *Atopobium*, *Prevotella*, *Corynebacterium*, *Gardnerella* Shared *Corynebacterium*, *Staphylococcus*, *Anaerococcus*, *Lactobacillus*, *Prevotella*	Male genital microbiome was more diverse compared to female Genital samples indicated big variability from an individual to another Several fluctuations in the diversity and composition of the genital microbiome associated with fertility/infertility
(Okwelogu et al., 2021)	To determine the microbiome compositions of the semen and vagina from couples seeking assisted reproductive health care, to investigate whether seminal microbiome differs substantially from the vaginal microbiome, and to identify bacterial taxa associated with positive IVF clinical outcomes	Cross-sectional	36 couples with infertility (Nigeria) Male 26-60 y Women 26-45 y Tx No	Semen aliquot Vaginal swab F/U No	Men *Lactobacillus*, *Gardnerella*, *Veillonella*, *Corynebacterium*, *Escherichia*, *Prevotella*, *Enterococcus, Megasphaera* Women *Lactobacillus*, *Prevotella*, *Gardnerella*, *Megasphaera*, *Olsenella*, *Sneathia* Shared many of the predominant genera (56%) *G. vaginalis*, *L. iners*, *L. japonicus*, *L. jensenii*	Seminal microbiome composition was more diverse but lower in bacterial concentrations compared to the vagina Significant association between the microbiome of semen and vaginal samples in couples with infertility Semen samples with positive IVF outcome were less diverse and significantly colonised by *L. jensenii* and *Faecalibacterium*, and significantly less colonised by *Proteobacteria* and *Bacteroidetes* phyla, and *Prevotella*

						Vaginal samples with positive IVF outcome were significantly colonised by *L. gasseri* and presented higher *Firmicutes*/*Baceroidetes* ratio
(Mehta et al., 2022)	To characterise penile microbiome composition over a 1-year period and to identify factors associated with penile microbiome composition over time	Prospective	218 heterosexual couples (Kenya) Men 26 (24-30) y Women 22 (20-25) y Tx No	Penile swab Cervicovaginal lavage F/U 12 months	Men *Corynebacterium*, *Streptococcus*, *S. sanguinegens*, *Finegoldia*, *Anaerococcus*, *L. iners*, *Prevotella*, *G. vaginalis*, *Veillonella* Women *L. iners*, *G. vaginalis*, *L. crispatus* Shared *Lactobacillus* spp., *G. vaginalis*	Penile microbiome composition was stable over a 1-year period and was influenced by circumcision status, sexual practices, female partner's vaginal CST and BV status, and men's HSV-2 status BV was positively associated with the relative abundance of numerous individual penile taxa
(Iniesta et al., 2022)	To determine the effect of a probiotic strain on the genitourinary dysbiosis	Interventional	17 couples with infertility and genitourinary dysbiosis (Caucasian) Men median 36 y Tx oral *Ligilactobacillus salivarius* PS11610 (10^9 CFU once daily for 6 months) Women median 35 y Tx oral *Ligilactobacillus salivarius* PS11610	Penile swab, semen aliquot Vaginal swab F/U 6 months	Men *Peptoniphilus*, *Finegoldia*, *Lactobacillus*, *Streptococcus*, *Corynebacterium*, *Staphylococcus*, *Campylobacter*, *Prevotella*, *Anaerococcus* Women *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Staphylococcus*, *Prevotella*	Male samples showed higher bacterial diversity than vaginal samples Post-treatment, the percentage of *Lactobacillus* in relation to the total bacterial counts increased in the vaginal microbiome At post-treatment, male urogenital microbiome showed slightly decreased pathogens and *Staphylococcus* spp. Post-treatment, shift from a proinflammatory to an anti-

			(10^9 CFU twice daily for 6 months)		Shared *Lactobacillus*, *Garnerella*, *Prevotella*	inflammatory profile of the couples at systemic level
(Koort et al., 2023)	To determine the potential impact of female and male partners' reproductive tract microbiome composition on ART outcome	Cross-sectional	97 couples with infertility (Estonia) Men 37.4 (25–58) y Women 34.1 (25–46) y Tx No 12 fertile couples (Estonia) Men 34.1 (22–42) y Women 32.3 (25–42) y	Semen aliquot Vaginal swab F/U No	Men *Lactobacillus*, *Acinetobacter*, *Prevotella*, *Corynebacterium*, *Campylobacter*, *Flavobacterium*, *Finegoldia*, *Porphyromonas* Women *L. crispatus*, *L. iners*, *Gardnerella*, *Atopobium*, *Bifidobacterium*, *Clostridium*, *Prevotella* Shared 96% of all detected OTUs (couples with infertility) and 65% (fertile couples) *Lactobacillus*, *Prevotellaceae*, *Bifidobacteriaceae*	Semen microbiome diversity is higher compared to vagina Reproductive microbial communities of couples with infertility were significantly more diverse and with different predominance patterns in comparison to fertile couples Couples with beneficial microbiome types had a significantly higher ART success rates compared to other couples Gram-negative anaerobes and microaerophiles associated negatively with ART success in both men and women
(Baud et al., 2023)	To investigate the composition of genital microbiome in infertile couples and its potential impact on infertility, to explore the potential interaction between male and female microbiome, to investigate whether	Cross-sectional	65 couples with infertility (Switzerland) Men reproductive age Women 25-4 y Tx No	Penile and semen swabs Vaginal swab F/U No	Men *Lactobacillus*, *Prevotella*, *Gardnerella*, *Corynebacterium*, *Staphylococcus*, *Porphyromonas*, *Peptoniphilus*, *Finegoldia*, *Campylobacter*, *Mobiluncus*	Vaginal samples had the highest bacterial load and male samples showed the highest diversity The paired vaginal and penis samples showed the lowest dissimilarity values compared to the vagina-semen

	the microbiome of one partner could influence the composition of the other partner's microbiome	Women *Lactobacillus*, *G.vaginalis*, *P. bivia*, *Atopobium* Shared *Lactobacillus*, *Prevotella*, *Staphylococcus*, *Ezakiella*	A slight impact of male microbiome on the female bacterial colonisation

ART: assisted reproductive technology; BV: bacterial vaginosis; CFU: colony forming unit; CST: community state type; F/U: follow-up; IVF: *in vitro* fertilisation; HSV: herpes simple virus; IUI: intrauterine insemination; OTU: operational taxonomic unit; Tx: treatment; y: years old

Concurrently, in vaginal samples, increased proportions of *L. gasseri* and decreased proportions of *Bacteroides* and other lactobacilli were observed in cases of positive IVF outcomes (Okwelogu *et al.*, 2021). Similarly, a positive outcome of intrauterine insemination was linked with an increased proportion of *L. crispatus* in the vagina, whereas no difference was detected in the semen (Amato *et al.*, 2020). In another study, women with BV or a vaginal microbiome dominated by *L. iners* or *L. gasseri* demonstrated reduced ART success rates compared to women with a *L. crispatus*-dominant or other lactic-acid-bacteria-predominant microbiome (Koort *et al.*, 2023). This finding corroborates previous research highlighting the protective role of *L. crispatus* in reproductive health (Srinivasan *et al.*, 2012; Koedooder *et al.*, 2019; Amato *et al.*, 2020). In men, those with a seminal microbiome dominated by *Acinetobacter* in combination with other bacteria had the highest ART clinical pregnancy rate, while the seminal microbiome dominated by Gram-negative anaerobic and/or microaerophilic bacteria such as *Prevotella*, *Porphyromonas*, *Dialister*, *Campylobacter* associated with poorer ART outcomes (Koort *et al.*, 2023). On the couple level, those who had beneficial microbiome types had superior ART success rate of 53% compared to the rest of the couples (25%) (Koort *et al.*, 2023). Interestingly, healthy couples seem to have lower microbial diversity than the couples undergoing ART (Koort *et al.*, 2023), meaning that an increased diversity in the reproductive microbiome may not necessarily be beneficial for fertility. Indeed, healthy vaginal microbiome is typically characterised by low diversity and dominance by one or few *Lactobacillus* species (Ravel *et al.*, 2011; Ma *et al.*, 2012; Onderdonk *et al.*, 2016), while there are conflicting results of the seminal microbial diversity and male health. Specifically, conditions like human immunodeficiency virus (HIV) and azoospermia are associated with lower microbial diversity (Hladik and McElrath, 2008; Liu *et al.*, 2014; Chen *et al.*, 2018), while prostatitis tends to correlate with increased

diversity (Mändar *et al.*, 2017). This is somewhat counterintuitive when compared to the general perception of the gut microbiome, where high diversity is considered as indication of good health (Clemente *et al.*, 2012; Lozupone *et al.*, 2012), underscoring the complexity of the reproductive microbiome's role in fertility.

Understanding the influence of the microbiome on reproductive functions becomes more complex due to the variability of these communities, which can be influenced by numerous factors including sexual activity, hormonal shifts, microbial treatments, and various other causes. One possible mechanism by which genital tract microorganisms can affect fertility is by inducing infection- or dysbiosis-related oxidative stress in both partners (Mändar *et al.*, 2013; Ahelik *et al.*, 2015; Altmäe and Kullisaar, 2022). Oxidative stress can damage sperm DNA, decrease sperm motility, and interfere with the normal function of the female reproductive tract, all of which are detrimental to fertility (Alahmar, 2019). Therefore, attention may be required to address these disturbances before undertaking ART procedures.

Microbiome modulation strategies. Several studies highlight the potential of microbiome modulation as a strategy for improving reproductive health in both men and women. A previous *in vitro* study investigated the potential impact of vaginal isolated microorganisms on sperm motility, where several vaginal bacteria, including *G. vaginalis*, *Staphylococcus aureus*, *S. agalactiae*, *E. coli*, and different *Lactobacillus* spp. effectively adhered to sperm and significantly reduced sperm motility and penetration in a viscous medium, suggesting a potential detrimental impact on fertility (Wang *et al.*, 2020). This work highlights the dual nature of *Lactobacillus*, since this genus has been positively associated with anti-inflammatory cytokines, possibly reducing the generation of pro-inflammatory cytokines (Kyongo *et al.*, 2012), while high adhesion of *Lactobacillus* spp. to sperm cells significantly reduced sperm functions, which could

negatively impact reproductive health (Wang *et al.*, 2020). It could be that with a good-quality semen with abundant spermatozoa the bacterial adhesion does not have noticeable effect on seminal parameters, while it might be pronounced in seminal samples with low spermatozoa counts. Nevertheless, probiotic interventions aimed at promoting a healthy vaginal microbiome dominated by *Lactobacillus* might mitigate the detrimental effects of pathogenic bacteria in sperm. If specific bacteria indeed compromise sperm motility, understanding and manipulating the vaginal microbiome might prove to be a novel strategy in fertility treatments.

The first intervention study of probiotic treatment performed in couples with infertility assessed the effect of a 6-month treatment with oral probiotic *Ligilactobacillus salivarius* PS11610 on the genital dysbiosis (Iniesta *et al.*, 2022). Oral intake of the probiotic resulted in the clearance of dysbiosis in 88.9% of the couples. Along the treatment, the vaginal microbiome mainly increased the abundance of *Lactobacillus* in relation to the total bacterial counts, while seminal microbiome displayed slightly lower levels of pathogens and staphylococci and changes in the microbial composition (Iniesta *et al.*, 2022). Further, the systemic immunological status in both partners was assessed, and a switch from pro-inflammatory to anti-inflammatory profile post-treatment was found (Wang *et al.*, 2020). Although as preliminary, altogether the intake of *L. salivarius* PS11610 slightly enhanced the rates of pregnancy and childbirth among 17 couples with unexplained infertility undergoing ARTs (Iniesta *et al.*, 2022).

Associations between the vaginal and penile microbiomes

The penile skin microbiome, like other skin microbiomes, is complex and diverse, consisting of various bacteria, fungi, and viruses. A healthy penile skin microbiome is

dominated by bacteria from genera like *Corynebacterium*, *Staphylococcus*, *Prevotella*, *Finegoldia*, *Peptoniphilus*, *Porphyromonas*, and *Anaerococcus* (Onywera *et al.*, 2020b), harbouring a richer but less abundant microbial community compared to the vagina (Baud *et al.*, 2023). However, the penile microbiome may have fewer regulatory factors or may be less susceptible to perturbations than vagina (Mehta *et al.*, 2022b). It has been shown that circumcision substantially modifies the penile skin microbiome, particularly by decreasing its α-diversity and reducing the presence of BV-associated genera and anaerobic bacteria (Gray *et al.*, 2009; Eren *et al.*, 2011; Nelson *et al.*, 2012; Liu *et al.*, 2013, 2015; Plummer *et al.*, 2021; Mehta *et al.*, 2022b). Changes in this microbiome can potentially influence the risk of urinary tract infections, STIs, and other conditions in the couple.

A recent longitudinal study revealed that the composition of the penile microbiome is stable over a one-year period in 50-60% of men (Mehta et al., 2022). The penile skin microbiome has been correlated to the vaginal microbiome in a number of studies with inconclusive results (Zozaya *et al.*, 2016; Plummer *et al.*, 2018, 2021; Mores *et al.*, 2021; Mehta *et al.*, 2022b, 2022a; Baud *et al.*, 2023). The penile skin bacterial communities from couples with BV were significantly more similar to their female partner's vaginal communities than to the vaginal communities of non-partner women in the study (Zozaya *et al.*, 2016), being in line with research where BV in women has been positively associated with the relative abundance of numerous individual penile taxa (Mehta *et al.*, 2022b; Baud *et al.*, 2023). However, these associations between the vaginal and penile microbes are often derived from studies following treatment for BV, limiting our understanding of these relationships in healthy states.

A pairwise comparisons of microbial composition between vagina-penis and vagina-semen in couples with infertility showed that the vaginal and penile samples were more

similar than the vaginal and semen samples, and that the penile and semen samples displayed higher similarity when they were collected from the sample individual compared to the same sample types from different men (Baud *et al.*, 2023). This study concludes that the male microbiome has a minimal influence on the bacterial colonisation in females, although the authors acknowledge that the information of sexual activity was missing.

Another study collected daily vaginal and penile specimens from a female participant and her male sexual partner through 3 weeks, where a dynamic interaction between the microbiomes of sexual partners were characterised, revealing the potential for microbial transmission and alteration of microbial composition following regular sexual intercourse (Mores *et al.*, 2021). The study revealed an increase in the abundance of *Streptococcus mitis* post-coitally, suggesting sexual transmission of this microorganism. *S. mitis* is a bacterium usually associated with the oral cavity but has also been detected in the urogenital tract (Mitchell, 2011; Onderdonk *et al.*, 2016). In fact, the researchers found the *S. mitis* strains from the female partner's urogenital tract to be closely related to one strain from the male partner's oral cavity (Mores *et al.*, 2021), highlighting the possible oral-to-genital bacterial transmission. A case report on a woman with no previous vaginal and oral infection but developed recurrent vaginal problems and gingivitis after starting a relationship, revealed lower *Lactobacillus* in the vagina and higher *Corynebacterium* levels in penis (Carda-Diéguez *et al.*, 2019). Intriguingly, other studies indicate that a *Corynebacterium*-dominated and low-diversity penile microbiome might have beneficial health associations for men and their female partners (Onywera *et al.*, 2020a; Mehta *et al.*, 2022b). Despite substantial progress in characterising these microbial sites, the dynamics of microbial sharing between the penile skin and vagina needs further investigation.

Microbial modification of penile skin via BV treatment. Several studies have focussed on the treatment of male counterparts when their female partners are experiencing recurrent BV. The rationale for this approach is based on the fact that sexual transmission may play a role in recurrent BV, since BV-associated bacteria have been detected in different parts of the male genitourinary tract (i.e., penis, urethra, urine, and semen) (Plummer *et al.*, 2021). Despite the logical rationale of treating both partners in cases of recurrent BV (Plummer *et al.*, 2018), previous randomised controlled trials (RCTs) that targeted male partner microbes have not successfully decreased the recurrence of BV (Swedberg *et al.*, 1985; Vejtorp *et al.*, 1988; Mengel *et al.*, 1989; Moi *et al.*, 1989; Vutyavanich *et al.*, 1993; Colli *et al.*, 1997). Nonetheless, a recent review presented that the reliability of the evidence from these RCTs ranged from low to very low (Amaya-Guio *et al.*, 2016). Notably, none of the past trials evaluated the use of topical antibiotics for men. However, other authors hypothesised that while oral antibiotics may effectively target bacteria from internal areas of the male reproductive tract, cutaneous bacteria colonising the penis may be more effectively eradicated with topical antibiotics (Plummer *et al.*, 2018). Therefore, it seems feasible that a combination of oral and topical antimicrobial treatments could be necessary to eliminate BV-associated bacteria.

In a previous exploratory study, the female participants diagnosed with BV received oral or intravaginal antibiotic (i.e., standard BV therapy) while their male partners received combined topical and oral antimicrobial treatment with both treatments lasting for 7 days (Plummer *et al.*, 2018). The obtained results showed that while the immediate outcome was promising, with reductions in BV-associated bacteria and increased *Lactobacillus* colonisation, BV-associated bacteria re-emerged in the penile microbiome after 3 weeks and the beneficial effects did not sustain in the long term (Plummer *et al.*, 2018). As the next step, the same antibiotic intervention in women with BV and their male partners was

carried out and followed up in a 12-weeks period of time (Plummer *et al.*, 2021). Again, the combined oral and topical treatment in men aimed to address multisite carriage of BV-associated bacteria. At 12 weeks post-treatment, the majority of women experienced suppression of BV-associated bacteria and an increase in *Lactobacillus* spp., suggesting that a male's combined therapy could be more effective than oral treatment alone. However, the male genital microbiome did not significantly differ from baseline after 12 weeks, with BV-associated bacteria re-emerging at male sites, cutaneous penile and urethra (Plummer *et al.*, 2021). These works bring to light the challenges in managing recurrent BV and underscores the importance of considering both partners in the treatment strategy. Despite the re-emergence of BV-associated bacteria in men over time, the beneficial effects seen in women suggest that treating men may still play a role in managing recurrent BV (Plummer *et al.*, 2018, 2021). However, these studies also highlight gaps in our understanding of the male genital microbiome and the role it plays in BV recurrence. While it seems logical to treat both partners in cases of recurrent BV, the appropriate treatment strategy and the factors that influence treatment success in men remain unclear.

Microbiome in same-sex partners

Same-sex couples also experience an exchange of microbial communities during sexual intercourse, albeit with different implications due to the anatomical distinctions. Men who have sex with men (MSM) have been found to harbour unique rectal microbiota compared to different-sex men (Noguera-Julian *et al.*, 2016), which might influence the susceptibility to HIV infection and other STIs. Also, the seminal microbiome can vary substantially between men of different sexual preferences, as rectal microbiome of MSM engaging in condomless receptive anal intercourse showed *Prevotella*-rich microbiome

with decreased diversity (Kelley *et al.*, 2017; Armstrong *et al.*, 2018), which could have different consequences for men's health.

Among female same-sex couples, shared vaginal microbiota is common and has been linked to BV, demonstrating a higher incidence of BV (Marrazzo *et al.*, 2010; Bradshaw *et al.*, 2014). Additionally, female long-term partners seem to share *Lactobacillus* strains, which could be beneficial to the health of both partners (Marrazzo *et al.*, 2009). In contrast, women who continually changed partners were more likely to have BV (Vodstrcil *et al.*, 2015). These are the first studies in the field and the understanding of microbial interactions and colonisation among same-sex partners clearly warrants more research.

Diseases related to sexual intercourse

A notable example of the interaction and mutual influence between the seminal and vaginal microbiotas is observed in the development of various diseases. These microbial interactions within host tissues and organs can have significant implications for fertility and the chances of achieving a successful pregnancy, leading to impairments in reproductive function. Therefore, these diseases are an important focus of study. According to the WHO, a sexually transmitted disease (STD) is primarily transmitted through unprotected vaginal, anal, or oral sex from one partner to another. However, it can also be transmitted through blood and from mother to child during pregnancy, childbirth, or breastfeeding (Riegler *et al.*, 2023). The STDs can be caused by viruses, bacteria, or parasites. The most common bacterial STDs include chlamydia (*C. trachomatis*), syphilis (*Treponema pallidum*), gonorrhoea (*Neisseria gonorrhoeae*), and mycoplasma (*Mycoplasma genitalium*). Viral infections include human papillomavirus

(HPV), herpes (herpes simplex virus [HSV]), HIV, and Hepatitis B. Some STDs like trichomoniasis are also caused by parasites (*Trichomonas vaginalis*) (Tuddenham *et al.*, 2021, 2022). Knowing which microorganism is causing the infection is crucial for appropriate treatment strategies. Viral infections typically cannot be completely cured but can be managed and their symptoms alleviated. On the other hand, bacterial infections offer a broader range of treatment possibilities, as they can often be effectively treated with antibiotics. Therefore, accurately identifying the specific microorganism responsible for the infection is vital in determining the most appropriate and effective course of treatment (Plummer *et al.*, 2021; Del Romero *et al.*, 2023).

HIV is the causative agent of acquired immunodeficiency deficiency syndrome (AIDS), a sexually transmitted disease with a high prevalence despite previous advancements (Masson *et al.*, 2015). It is understood that microabrasions during sexual intercourse in both male and female genital tracts serve as the primary route for HIV to access its target cells, as they degrade the protective barrier formed by the epithelia (McCoombe and Short, 2006; Mehta *et al.*, 2020a). Predisposing factors such as inflammatory reactions and an altered state of the microbiota have also been identified (Masson *et al.*, 2015; Jewanraj *et al.*, 2020). Specifically, vaginal dysbiosis (i.e., BV) and the changes induced by semen on the vaginal microbiota have been found not only to disrupt the microbiota barrier but also to recruit immune system cells, which are susceptible to HIV infection (Anahtar *et al.*, 2015; Cherne *et al.*, 2020; Mtshali *et al.*, 2021). Furthermore, anaerobic microorganisms present within the foreskin have been shown to increase the likelihood of infection in the male genital tract during sexual intercourse (Prodger *et al.*, 2014). The role of microorganisms in HIV transmission still requires further investigation, as a favourable seminovaginal microbiota may potentially reduce viral entry to some extent (Jewanraj *et al.*, 2020).

Although STDs are widely recognised, there exist other conditions that can emerge because of unprotected sexual activity but do not fall under the definition of STDs. These conditions are referred to as "sexually enhanced diseases", which individuals can develop even without engaging in intercourse. However, participating in sexual intercourse can substantially heighten the probability of acquiring these diseases due to the factors such as the composition of their partner's microbiota, the characteristics of bodily fluids, or physical trauma sustained during sexual activity. Among the sexually enhanced diseases, specifically related to women, the BV is a commonly occurring vaginal condition that is linked to various obstetric and gynaecological complications and has substantial implications for healthcare costs. The aetiology of BV is not fully established; however, it has been suggested that it is transmissible, and that *G. vaginalis* may be an etiological agent (Eren *et al.*, 2011). Gynaecologic evaluation includes the Nugent score and Amsel criterion assessment. It involves evaluating the presence of specific bacterial morphotypes. Large Gram-positive rods (*Lactobacillus* morphotypes) are assessed for a decrease in quantity, with a score ranging from 0 to 4. Small Gram-variable rods (*G. vaginalis* morphotypes) are also evaluated and scored from 0 to 4. Additionally, curved Gram-variable rods (*Mobiluncus* spp. morphotypes) are considered and scored from 0 to 2. A total score of 7 to 10 indicates the presence of BV without requiring a culture-based diagnosis (Sha *et al.*, 2005). In women with BV, the composition of the vaginal microbiota is characterised by a decrease in *Lactobacillus* spp. and an increase in specific anaerobic bacteria collectively referred to as BV-associated bacteria. These bacteria include *Gardnerella* spp., *A. vaginae*, *Prevotella* spp., *Sneathia* spp., and others (Plummer *et al.*, 2021). Sexual activity is clearly linked to the development of BV but likely through a more complex mechanism than some other STIs. Some have hypothesised that the change in vaginal pH resulting from semen is what drives the shift

in microbiota that results in BV (Mitchell *et al.*, 2012). When BV is linked to sexual activity, it typically arises due to an alkalization of the vaginal microbiota caused by semen's pH and the transmission of bacteria from the woman's perianal region (Verstraelen *et al.*, 2010). Additionally, the transfer of *G. vaginalis* from the seminal to the vaginal microbiota may contribute to this condition (Vodstrcil *et al.*, 2017). In either case, sexual intercourse diminishes the abundance of *L. crispatus*, compromising the woman's defence and making it more susceptible to conditions like BV and different STDs (Verstraelen *et al.*, 2010; Vodstrcil *et al.*, 2017). It is interesting to observe that circumcision has been shown to reduce the abundance of anaerobic bacteria in the penile microbiota and has been associated with a reduced risk of BV in female partners (Mehta *et al.*, 2020b). Therefore, it is probable that circumcision impacts not only a woman's risk of BV recurrence, but also the effectiveness of male partner treatment strategies (Plummer *et al.*, 2021). Although male circumcision reduces BV-associated bacteria on the penis and decreases BV in female partners, the link between the penile microbiota and female partner BV is not well understood (Liu *et al.*, 2015). Another STDs with special relevance in females are the Candidiasis vulvovaginitis, a disease caused by the proliferation of fungi of the genus *Candida* in the vaginal microbiota (Rolo *et al.*, 2020). *Candida albicans* is usually the main cause of the infection, although other species such as *Candida krusei* and *Candida parapsilosis* can also cause it (Zaman *et al.*, 2022). *Candida* spp. can be found in the vaginal microbiota without causing harm, but as an opportunistic pathogen, it can take advantage of situations as physiological imbalance to proliferate (Ventolini *et al.*, 2006). Therefore, the development of candidiasis vulvovaginitis may be due to genetic and/or environmental factors, as well as the use of antibiotics (Ventolini *et al.*, 2006) and sexual intercourse (Mendling *et al.*, 2020). It can be transmitted directly from the seminal microbiota or the composition of semen can favour the growth of

Candida strains already present in the vaginal microbiota (Mendling *et al.*, 2020). A study demonstrated that the semen can promote this disease by presenting factors that stimulate the growth of the fungus, particularly favouring the development of hyphae (Rolo *et al.*, 2020). It was also observed that the proliferation of *C. albicans* was decreased, at least partially, by an increase in semen viscosity. These data still need to be cross-checked with other similar studies, as previous research has shown that semen presents antifungal factors (Edström Hägerwall *et al.*, 2012), however these do not appear to be effective against *Candida* growth (Rolo *et al.*, 2020).

Regarding STIs in males, the urethritis is one of the most frequent complications, classified as gonococcal (GU) or non-gonococcal (NGU) according to the presence or not of *N. gonorrhoeae*. The main microorganisms responsible for NGU are *C. trachomatis*, *M. genitalium*, *T. vaginalis*, *Ureaplasma* spp., and HSV type 1 and 2; however, it is estimated that the aetiology is unknown in up to 40% of NGUs when PCR is the sole diagnostic method (Franco-Acosta *et al.*, 2022). *Haemophilus influenzae* and *H. parainfluenzae*, which colonise the healthy upper respiratory tract, have been reported as rare agents responsible for NGU, especially among MSM (Franco-Acosta *et al.*, 2022). Altogether various microorganisms that include bacteria, viruses, and fungi participate in the infections/diseases related to sexual intercourse, however the detailed mechanisms of their role need to be established.

CONCLUSION

The human vaginal microbiota is better characterised than the seminal microbial composition, while both play an important role in the host protection and participate in reproductive functions, nevertheless the detailed mechanisms need to be established.

During unprotected sexual intercourse, the vaginal and seminal microbes mix and influence each other, forming the seminovaginal microbiota, which has significant effects on the health and reproductive success of the couple. Understanding the composition and dynamics of the seminovaginal microbiota is crucial for addressing infertility, adverse pregnancy outcomes, BV, and STDs/STIs. Furthermore, the sexual exchange of microorganisms between partners can have both beneficial and detrimental effects on the health of couples, and an in-depth understanding of these microbial exchanges could pave the way for interventions to promote reproductive health and prevent infections.

Currently, there is limited knowledge about the microorganisms shared by the couple after sexual intercourse and their function in modulating couple's microenvironment. Exposure to semen can cause changes in the microbial composition, leading to vaginal dysbiosis, infertility, and inflammatory reactions that affect sperm count. The stability of the seminovaginal microbiota against the impact of semen is a key factor to consider in ART.

While previous studies have focussed on the vaginal and seminal microbiotas separately, future research should approach them as a complementary microbiota, recognising their interconnection and the significant role they play in reproductive health and success. Overcoming the limitations of current research and adopting the concept of couple's microbiome as holistic concept would provide a better understanding of the seminovaginal microbiota and its implications, leading to improved strategies for addressing reproductive challenges and promoting optimal reproductive outcomes.

SUPPLEMENTARY MATERIAL

In addition, the supplementary files can be downloaded in this link: https://osf.io/um34n/?view_only=2781e441e845423ba1b167e3c7274383

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Study IV: Analysing endometrial microbiome: methodological considerations and recommendations for good practice

ABSTRACT

There is growing evidence that the upper female genital tract is not sterile, harbouring its own microbial communities. However, the significance and the potential effect of endometrial microorganisms on reproductive functions remain to be fully elucidated. Analysing the endometrial microbiome, the microbes, and their genetic material present in the endometrium, is an emerging area of study. The initial studies suggest it is associated with poor reproductive outcomes and with different gynaecological pathologies. Nevertheless, studying a low biomass microbial niche as is endometrium, the challenge is to conduct well-designed and well-controlled experiments in order to avoid and adjust for the risk of contamination, especially from the lower genital tract. Herein, we aim to highlight methodological considerations and propose good practice recommendations for future endometrial microbiome studies.

INTRODUCTION

Few, if any, tissues in our body are totally sterile, with most having a coevolved microbiota, i.e., community of microorganisms that inhabits a defined site. Indeed, recent studies have sequenced hyper-variable regions of the bacterial 16S rRNA gene and have identified a unique endometrial microbiome, i.e., microorganisms and their genomes (Baker *et al.*, 2018; Benner *et al.*, 2018; Koedooder *et al.*, 2019). The uterine (endometrial) microbiome is considered as a low microbial biomass site since the estimation of the uterine bacterial load is 100–10 000 times lower than that of the vagina (Baker *et al.*, 2018).

Endometrial microbiome composition has been associated with various gynaecological diseases such as endometriosis, chronic endometritis, dysfunctional endometrial bleeding, endometrial cancer or hyperplasia, and poorer outcomes in assisted reproduction (Molina *et al.*, 2020) (see **Table 1** for all studies). Especially, the potential implications for human reproduction have sparked research in a previously overlooked infectious cause of infertility. However, there is in fact only one study to date that has detected a statistically significant difference in microbiome profiles between successful and unsuccessful reproductive outcomes (Moreno *et al.*, 2016). Other studies have not detected any significant associations between endometrial microbiome and reproductive outcomes (Franasiak *et al.*, 2016; Verstraelen *et al.*, 2016; Kyono *et al.*, 2018, 2019; Liu *et al.*, 2018; Wee *et al.*, 2018; Hashimoto and Kyono, 2019; Carosso *et al.*, 2020; Riganelli *et al.*, 2020). The study by Moreno *et al.* analysed 35 women with infertility undergoing IVF and detected *Lactobacillus* dominance in the uterus. Interestingly, non-*Lactobacillus*-dominated microbiome was associated with decreased implantation, pregnancy, and live birth rates among infertile women undergoing IVF (Moreno *et al.*, 2016). In most of the studies, the endometrial sample was obtained transcervically, which

is prone to the bacterial "contamination" from the lower genital tract. Notably, studies that obtained endometrial biopsies from hysterectomy (Winters *et al.*, 2019), laparoscopy (Chen *et al.*, 2017), and/or during caesarean section (Leoni *et al.*, 2019; Younge *et al.*, 2019) (lowering the contamination risk from the vagina and cervix) conclude that *Lactobacillus* does not dominate the uterine cavity, and bacteria such as *Pseudomonas*, *Acinetobacter*, *Vagococcus*, and *Sphinogobium* constitute a notable fraction of the endometrial microbiome, contradicting the findings of *Lactobacillus*-dominance in the uterus in studies using transcervical sampling method (Moreno *et al.*, 2016; Kyono *et al.*, 2018; Hashimoto and Kyono, 2019). Even more, 40% of the endometrial samples collected from abdominal hysterectomy did not present any detectable uterine microbiome above the negative controls (Winters *et al.*, 2019), which adds to the ongoing debate whether there is in fact a unique endometrial microbiome in all women, and whether the detected bacterial sequences refer to tourists, residents, invaders, or contamination.

Difficulty in establishing the endometrial core microbiome is further hindered by the fact that the original works performed so far are barely comparable (see **Table 1**), with different study protocols and several other limitations, leaving us far from drawing any conclusions on the composition and role of the microbial communities in the endometrium in health and disease. It is important to be meticulous in designing, analysing, and interpreting studies of the endometrial microbiome, as many factors starting from patient selection, sampling methods and handling, laboratory experiments, statistical analyses, and other confounding factors can lead to potential bias and hamper study validity, reliability, and generalisability (Molina *et al.*, 2020). There is an urgent need for standardised methodologies and data processing of the obtained results in the fast-growing field of endometrial microbiome in order to improve comparability of

studies and facilitate meta-analyses. With this review, we give an overview of the methodology that is currently used to study the endometrial microbiome and highlight factors that can influence studies in niches with low microbial abundance, as is the endometrium. Furthermore, we aim to provide recommendations for conducting well-designed, clinically relevant studies with detailed metadata in order to adequately analyse and explore the clinical implications of reproductive tract exposure to microorganisms and to ensure that quality research in endometrial microbiome studies is undertaken.

CONSIDERATIONS ON STUDY DESIGN

Designing an experiment that generates meaningful data is the first important step. Differences in microbial community structure, composition, and genetics or function between separate cohorts (i.e., case-control or cross-sectional studies) or over time (i.e., longitudinal studies) can be studied in the context of endometrial microbiome. Prospective studies should aim to collect the samples at identical time points as well as sequential samples from the same individual at different time points (Knight *et al.*, 2018). For instance, in the gut, microbial community instability rather than the specific taxa present at a single time point has been considered as a predictor of a disease (Knight *et al.*, 2018).

Adequate sample size is another important point. Majority of the endometrial microbiome studies performed to date are under-powered, including on average 30–60 participants (**Table 1**). Statistical power and effect size analyses are a challenge, and proper methods such as Dirichlet Multinomial, PERMANOVA, or random forest analyses should be applied in order to determine technical variability and to obtain true biological results (Knight *et al.*, 2018; Qian *et al.*, 2020).

Table 1. Microbiome studies in human endometrium

Study	Sample (N); Age (y); Ethnicity/Race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Mitchell *et al.* 2015	Hysterectomy patients (N=58); avg 43±7y; Caucasian, African American, Hispanic	Endometrial swab from excised uterus (-80ºC)	NR	MoBio Bacteremia DNA Isolation Kit; qPCR for 12 vaginal bacterial species and broad-range 16S rRNA gene qPCR	*Lactobacillus iners, Prevotella* spp., *L. crispatus*	NR	95% of women had endometrial colonisation with bacteria Quantity of bacteria in uterus was lower than that in the vagina Detection of bacteria in the uterus was not associated with inflammatory immune response
Franasiak *et al.* 2016	IVF patients (N=33); avg 35.9y; Caucasian, Asian, African American, Hispanic	Transfer catheter (snap-frozen and stored at -20ºC after cell lysis)	Negative controls from reagents; Positive control: *Escherichia coli*	Cell lysis and DNA purification (Agencourt AMPure® XP Reagent); Ion PGM™ sequencing (V2-4-8, V3-6, 7-9)	*Flavobacterium*, *Lactobacillus*	NR	No significant differences in endometrial microbiome between successful and unsuccessful IVF

Verstraelen *et al.* 2016	RIF (N=11); RPL (N=7); RIF+RPL (N=1); avg 32y; Caucasian	Tao Brush™ Endometrial Sampler (-80ºC)	Disinfection of the cervical surface and external os	Mechanical cell lysis and DNA purification; Illumina MiSeq® (V1-2)	*Bacteroides xylanisolvens, B. thetaiotaomicrom, B. fragiles, B. vulgatus, B. ovatus, Pelomonas, Betaproteoacteria, Escherichia/Shigella, Chitinophagaceae*	Supplementary material	The uterine core microbiome composed of three main bacterial phyla: *Proteobacteria, Firmicutes* and *Bacteroidetes*
Fang *et al.* 2016	EP (N=10); 34.4±2.4y; EP/CE (N=10); avg 35.2±1.8y; Control (N=10); 30.9±1.6y; Chinese	Uterine swab (-80ºC)	Vaginal and cervical canal disinfection	TIANamp Swab DNA Kit; Illumina MiSeq® (V4)	*Lactobacillus, Enterobacter, Pseudomonas, Euryarchaeota* (Archaea)	PRJEB9626 deposited in ENA	Bacterial populations in uterus greatly differed from vaginal microbiome Low levels of Archaea were detected in all samples Women with EP and EP/CE showed different endometrial microbiome composition compared to controls
Khan *et al.* 2016	Endometriosis (N=32); 21–47y; Control (N=32); 21–54y; Japanese	Seed swab (-80ºC)	NR	Ultra Clean PowerSoil® DNA Isolation Kit; Illumina MiSeq® (custom primers)	*Lactobacillacae, Streptococcaceae, Staphylococaceae, Enterobacteriaceae, Moraxellaceae*	NR	*Lactobacillaceae* were decreased and *Streptococcaceae* and *Moraxellaceae* increased in women with endometriosis GnRH treatment influenced bacterial proportions in uterus Slight increase in the microbial colonisation during the menstrual phase

Walther-António *et al*. 2016	Endometrial cancer (N=17); avg 64y; Endometrial hyperplasia (N=4); avg 54y; Benign condition (N=10); avg 44.5y; Caucasian	Endometrial swab, uterine scrapes and uterine biopsies from excised uterus at hysterectomy (-80ºC)	Negative controls: air culture during sample collection and controls of DNA extraction and microbiome enrichment processes	MoBio PowerSoil Kit; Illumina MiSeq® (V3-5)	*Shigella, Barnesiella, Staphylococcus, Blautia*, *Parabacteroides*	SRP064295 deposited in SRA repository	Significant subject-specific correlations in endometrial microbiome distribution Existence of structural microbiome shift in the cancer and hyperplasia cases Higher diversity in cancer cohort Detection of *A. vaginae* and *Porphyromonas* sp. 99% matching *P. somerae* as predictor of endometrial cancer Significant effects of collection type in profiling the microbiome
Moreno *et al.* 2016	IVF (N=35); 25–40y; Control (N=35); 18–35y; Spanish	Endometrial fluid with Wallace Classic transfer catheter (snap-frozen, -80ºC)	Cervix cleaning with a cotton swab and mucus aspirated	Extra MagNA Pure compact nucleic acid isolation Kit I; Pyrosequencing on Roche 454 (V3-5)	*Lactobacillus, Gardnerella, Bifidobacterium, Streptococcus*, *Prevotella*	SRP078557 deposited in SRA repository	Different bacterial communities detected between uterus and vagina Endometrial microbiome was highly stable throughout menstrual cycle Non-*Lactobacillus* dominance was associated with significant decrease in implantation, pregnancy, ongoing pregnancy and live birth rates in IVF
Miles *et al*. 2017	Hysterectomy patients (N=8); 41–57y; North American	Endometrial swab from excised uterus (-80ºC)	Positive and negative controls (not specified)	Pyrosequencing on Roche 454 (V1-3)	*Lactobacillus, Acinetobacter, Blautia, Corynebacterium*, *Staphylococcus*	NR	Bacteria identified in 95% of endometrial samples *Lactobacillus* was highly abundant Microbial communities were highly related across the uterine and cervical samples and patients
Tao *et al.* 2017	IVF (N=70); avg 36.2y; Caucasian, Asian, African,	Wallace Classic transfer catheter (NR)	Negative control: blank extraction control; Positive controls: different	Genomic DNA isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Corynebacterium* spp.,	NR	*Lactobacillus* was detected in all patients Provide method to study ultra-low amount of bacteria

	American, Hispanic		concentrations of mock communities		*Streptococcus*, *Staphylococcus* spp., *Bifidobacterium* spp.		
Cregger *et al.* 2017	Endometriosis (N=6); pre-menopausal; Control (N=3); North American	Uterine wash (-80ºC)	NR	Power Soil® DNA Isolation Kit; Illumina MiSeq® (V3-5)	*Lactobacillus* spp., *Barnesiella*, *Flavobacterium*, *Pseudomonas*	PRJNA387551 deposited in SRA repository	Uterine and cervical bacterial communities were significantly different Uterine microbiome was not significantly different across endometriosis stages
Chen *et al.* 2017	Various conditions not known to involve infection (N=95); 22–48y; Asian	Nylon flocked swabs at laparoscopy or laparotomy (snap-frozen, -80ºC)	Negative controls: some reagents (PBS, physiological saline and ultrapure water), diluent negative controls, dry sterile swabs rubbed on patient's preoperative skin area and surgeon's gloves	Chloroform:isoamyl alcohol extraction; Ion Torrent PGM™ (V4-5); conventional culturing methods and qPCR to detect 4 *Lactobacillus* spp.	*Pseudomonas, Acinetobacter, Vagococcus*, *Sphingobium, Comamonadaceae*, *Lactobacillus*	PRJEB16013 and PRJEB21098 deposited in ENA	Distribution of bacteria in the endometrial samples taken through the cervical os showed high similarity to that taken during surgery Upper reproductive tract showed higher bacterial diversity and lower bacterial biomass than vaginal microbiome Differences in the uterine microbiome were identified between menstrual phases Live bacteria detected in the upper reproductive tract
Wee *et al.* 2018	Infertile (N=6); avg 37.6y; Control (N=5); avg 42.6y; Australian	Endometrial curettage at hysteroscopy (-80ºC in RNALater)	Vagina treated with betadine antiseptic solution; Negative controls for lysis, extraction, and PCR	DNeasy Blood and Tissue Kit; Illumina MiSeq® (V1-3); qPCR to detect *Ureaplasma* spp.	*Lactobacillus, Bifidobacterium*	PRJEB18626 deposited in SRA repository	Endometrial microbiome differed from that in the lower reproductive tract, having generally lower relative abundance

Pelzer *et al.* 2018	Menorrhagia or dysmenorrhea (N=57); 14–52y; Control virgo intacta (N=3); Australian	Endometrial curettage collected at hysteroscopy or laparoscopy (NR)	Perineum, vagina and ectocervix were disinfected with povidone iodine	QIAmp Mini DNA extraction Kit; Pyrosequencing on Roche 454 (V5, V8)	*Lactobacillus*, *Gardnerella vaginalis, Veilonella, Prevotella*, *Sneathia*	NR	Endocervix and endometrium showed different microbiome Endometrial microbial communities were different between women with menorrhagia and dysmenorrhea Microbial communities were significantly different between menstrual phases
Kyono *et al.* 2018	IVF (N=79); avg 37±4.2y; Infertile non-IVF (N=23); avg 33.2±3.6y; Control (N=7); avg 36.6±6.6y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucous from cervix; Negative controls: blank control of clean water; Positive control: Zymo-BIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Gardnerella, Streptococcus, Atopobium, Bifidobacterium, Sneathia, Prevotella*, *Staphylococcus*	NR	Endometrial microbiome of healthy women was highly stable throughout menstrual cycle Considerable percentage of non-*Lactobacillus* dominated microbiome was found in the endometrium of infertile women Contaminants: *Acinetobacter*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Methylobacterium*, *Pseudomonas, Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*
Moreno *et al.* 2018	CE undergoing IVF (N=13); 21–53y	Novak curette connected to a 20-mL syringe (frozen)	Negative controls to detect any contamination from reagents; Positive control: *E. coli* DNA	QIAamp Cador Pathogen Mini Kit; Ion Torrent S5 XL (V2-4-8, V3-6, 7-9); qPCR to detect the 9 most common bacteria responsible for causing CE	*Lactobacillus*, *Streptococcus*, *Gardnerella*, *Bifidobacterium, Magasphaera, Parvimonas, Prevotella, Propionibacterium*, *Veillonella*	NR	16S rRNA gene sequencing and qPCR were effective techniques to discriminate between positive and negative cases of CE
Li *et al.* 2018*	Surgery for non-infectious condition	Endometrial swab at laparoscopy or laparotomy	Negative controls: some reagents (PBS, physiological saline and ultrapure water),	Chloroform:isoamyl alcohol extraction; BGISEQ-500	*Pseudomonadaceae, Propionibacteriaceae,*	PRJEB24147 deposited in SRA repository	Presence of intra-individual continuum of microorganisms Endometrial microbiome analysis using shotgun

	(N=3); avg 28.3y; Asian	(snap-frozen, -80ºC)	diluent negative controls, dry sterile swabs rubbed on patient's preoperative skin area and surgeon's gloves		*Streptococcaceae*, *Moraxellaceae*		metagenomics requires greater depth and more samples to meet the full coverage scale in sequencing
Liu *et al.* 2018	RPL (N=25); avg 35.2y; Chinese	Endometrial fluid collected using double-lumen ET catheter and endometrial tissue with Pipelle (-80ºC)	Removal of excessive vaginal secretions; Negative controls: collection medium blank and swabs exposed to the air of the clinic	QIAmp DNA Mini Kit plus Triton-X, mutanolysin and proteinase K; Illumina MiSeq® (V4)	*Lactobacillus*, *Stenotrophomas*, *Gardnerella, Bifidobacterium, Atopobium*, *Prevotella*, *Megasphaera*, *Staphylococcus*, *Escherichia*	NR	Microbial composition in the endometrial fluid did not fully reflect that of the endometrial tissue
Kyono *et al.* 2019	IVF (N=92); avg 37±4.1y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucus from cervix	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Atopobium, Bifidobacterium, Gardnerella, Megasphaera, Sneathia, Prevotella, Staphylococcus*, *Streptococcus*	NR	Microbial composition gradually changed along the upper reproductive tract Recovering *Lactobacillus* dominance might benefit implantation in IVF Percentage of endometrial *Lactobacillus* >80% might be favourable for embryo implantation
Kitaya *et al.* 2019	RIF (N=28); avg 38.7±3.2y; Control undergoing IVF (N=18); avg 37.6±4.2y; Asian	Endometrial fluid collected using MedGyn Pipette IV (stabilising medium)	Perineum, vagina and cervix were cleansed with benzalkonium chloride solution; mucus was removed; Negative control: clean water; Positive control: Zymo-BIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Gardnerella*, *Streptococcus*, *Prevotella*	NR	Bacterial species in endometrial fluid and vaginal samples were similar within the same individual, however fluid had higher diversity and broader bacterial species Significant variation in community composition between the RIF and controls Contaminants: *Acidovorax*, *Acinetobacter*, *Chryseobacterium*, *Citrobacter*,

							Elizabethkingia, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Leptothrix*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Yersinia*
Winters *et al.* 2019	Hysterectomy patients (N=25); avg 45y; Italian	Endometrial swab from excised uterus (-80ºC)	Negative controls from reagents	QIAGEN DNeasy PowerLyzer PowerSoil Kit; Illumina MiSeq® (V4); qPCR to detect *L. iners* and *L. crispatus* and V1-2 16S rRNA gene	*Acinetobacter*, *Pseudomonas*, *Cloacibacterium*, *Comamonadacea e*	PRJNA543861 deposited in SRA repository	Bacterial profiles of the endometrium differed from those of the oral cavity, rectum, vagina and background DNA controls, but not of the cervix Endometrial microbiome was not dominated by *Lactobacillus* 40% of patients did not present detectable microbiome over contamination Contaminants: *Veillonella*, *Escherichia*, *Streptococcus*, *Acinetobacter*
Liu *et al.* 2019	Infertile CE (N=12); avg 35y; Control infertile without CE (N=118); avg 36y; Chinese	Endometrial fluid collected using double-lumen ET catheter (-80ºC)	Removal of excessive vaginal secretions; Negative controls: collection medium blank and swabs exposed to the air of the clinic; Positive control: microbial mock community HM-277D	QIAmp DNA Mini Kit plus Triton-X, mutanolysin and proteinase K; Illumina MiSeq® (V4)	*Lactobacillus*, *Dialister*, *Bifidobacterium*, *Prevotella*, *Gardnerella*, *Anaerococcus*	PRJNA530321 deposited in SRA repository	CE associated with a significantly higher abundance of certain bacterial taxa in uterus Median relative abundance of *Lactobacillus* in CE group was lower than in non-CE group
Younge *et al.* 2019	Caesarean delivery (N=10); North American	Endometrial swab from the lining of the anterior uterine wall at	Negative controls: no DNA template for each extraction kit and PCR	Bead-beating and commercial extraction kits; Illumina MiSeq® (V4)	*Escherichia*, *Acinetobacter*, *Lactobacillus*, *Bacillus*,	PRJNA557826 available at NCBI BioProject	Endometrial microbiome did not differ from that of the vagina

		caesarean section (-80ºC)					
Garcia-Grau *et al.* 2019	Infertile (N=1); 37y; Spanish	Endometrial fluid collected using a Wallace transfer catheter (-80ºC in RNALater)	Cervix cleaning with a cotton swab and mucus aspirated	QIAamp DNA Blood Mini Kit; Ion Torrent S5 XL system (V2-4-8 and V3-6, 7-9); NextSeq 500 system; qPCR targeting clade-specific genes for *Gardnerella*	*Gardnerella vaginalis*, *Lactobacillus*, *Pseudoalteromonas*, *Bifidobacterium*, *Rhodanobacter*	PRJNA545633 deposited in SRA repository	Persistent *G. vaginalis* colonisation associated with reproductive failure (based on both 16S rRNA gene seq and metagenome seq)
Hashimoto and Kyono *et al.* 2019	IVF (N=99); avg 35.3±3y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucus from cervix; Negative control: clean water; Positive control: Zymo-BIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Gardnerella*, *Atopobium*, *Streptococcus*, *Lactobacillus*	NR	Pregnancy rate in IVF was comparable between *Lactobacillus* dominated microbiome and non-*Lactobacillus* dominated microbiome Species-level resolution might be required for identifying the true pathogenic bacteria and avoiding over-intervention against non-*Lactobacillus* dominated microbiome Contaminants: *Acinetobacter*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*
Leoni *et al.* 2019	Caesarean delivery (N=19); avg 32.3±7.6y; Caucasian	Endometrial biopsy at caesarean section (-80ºC)	Negative controls: fake extraction procedure and clean water	Fast DNA Spin Kit for Soil; Illumina MiSeq® (V5-6)	*Cutibacterium* (formerly *Propionibacterium*), *Escherichia*, *Staphylococcus*, *Acinetobacter*,	PRJNA557586 deposited in SRA repository	Bacterial composition could differ between different sites of the endometrium *Lactobacillus* can be found in the endometrium, but without being a part of the core microbiome

					Streptococcus, *Corynebacterium*		
Walsh *et al.* 2019	Endometrial cancer (N=66); avg 61.8±10.3y; Hyperplasia (N=7); avg 55±3.3y; Benign condition (N=75); avg 49.9±10.5y; Caucasian, Native American, Filipino, Chinese	Endometrial swab, uterine scrapes and uterine biopsies at hysterectomy (-80ºC)	Negative controls: during sample collection and controls of PCR amplification; Positive control	MoBio PowerSoil Kit; Illumina MiSeq® (V3-5)	*Shigella, Barnesiella, Staphylococcus, Blautia*, *Parabacteroides*	PRJNA481576 deposited in SRA repository	Each factor (menopause, BMI, vaginal pH) independently alters the endometrial microbiome *Porphyromas somerae* presence was the most predictive microbial marker of endometrial cancer
Moreno *et al.* 2020	IVF (N=1); 28y; Spanish	Endometrial fluid collected using a double lumen ET catheter (-80ºC in RNALater)	Cervix cleaning with a cotton swab and mucus aspirated Negative controls: blank controls from reagents; Positive control: *Escherichia coli* DNA	QIAamp DNA Blood Mini Kit; Ion Torrent S5 XL system (V2-4-8 and V3-6, 7-9); NextSeq 500 system	*Lactobacillus*, *Enterobacteriaceae*, *Streptococcus*, *Pseudomonas*, *Staphylococcus*	PRJNA514966 deposited in SRA repository	Taxonomic and functional differences in endometrial microbiome between spontaneous abortion and successful pregnancy in the same patient
Hernandes *et al*. 2020	Endometriosis (N=10); 18–50y; Control (N=11); Brazilian	Endometrial curettage (snap-frozen, -80ºC)	Negative controls: reagents from extraction procedure and PCR steps	DNeasy Power Soil Kit; Illumina MiSeq® (V3-4)	*Lactobacillus*, *Gardnerella*, *Streptococcus*, *Prevotella*	PRJNA546137 available at NCBI BioProject	Endometrium samples showed lower amount of relative reads and higher diversity than vaginal samples Endometriotic lesions demonstrated the highest microorganism diversity
Wei *et al*. 2020	Endometriosis (N=26); avg 31.47y; Control with conditions not known to	Endometial fluid collected using Huales Medical sampler at	Negative controls: saline	QIAamp DNA Mini Kit; Ion Torrent PGM™ (V4-5)	*Lactobacillus*, *Pseudomonas*, *Acinetobacter*, *Vagococcus*	Available from the corresponding author on	Existence of distinct community composition in the lower compared to the upper genital tract

	involve infection (N=11); Asian	laparoscopy (dry-ice, -80ºC)				reasonable request	Alteration in microbial community composition associated with endometriosis
Riganelli *et al*. 2020	Infertile (N=24); avg 37y; Italian	Endometrial biopsy collected using Pipelle covered by IUI catheter (-80ºC in Allprotect Tissue reagent)	External genitals and cervical canal disinfection; Negative controls: blank controls from reagents; Positive control: ZymoBIOMICS Microbial Community DNA Standard D6305	DNeasy Blood and Tissue Kit; Illumina MiSeq® (V4-5)	*Kocuria dechangensis*, *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia*, *Agrobacterium tumefaciens*, *Delftia tsuruhatensis*, *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Aerosakkonema fusiforme*, *Bacteroides ovatus*	PRJNA603234 deposited in SRA repository	Significant difference between vaginal and endometrial microbiome, with higher number of species and biodiversity in endometrium *Lactobacillus* was exclusively detected in the group that displayed unsuccessful IVF *K. dechangensis* was significantly predominant in endometrium among women who did not achieve pregnancy in IVF
Kadogami *et al*. 2020	RIF (N=392);avg 38.6y; Asian	Endometrial fluid collected using Pipette IV (stabilising medium)	Removal of the mucous from cervix; Negative controls: UltraPure™ Nase/RNase-Free Distilled Water; Positive control: ZymoBIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus*, *Bifidobacterium*, *Gardnerella*, *Atopobium*, *Streptococcus*, *Prevotella*	NR	*Lactobacillus*/*Bifidobacterium* presence associated with the stage of follicular development Increasing *Lactobacillus* colonisation during the luteal phase Combination of a vaginal probiotic suppository and antibiotics may represent an effective treatment for endometrial health Contaminants: *Acidovorax*, *Acinetobacter*, *Chryseobacterium*, *Citrobacter*, *Elizabethkingia*, *Escherichia*, *Flavobacterium*,

							Janthinobacterium, *Leptothrix*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Yersinia*
Carosso *et al*. 2020	IVF (N=15); avg 35.1y; Caucasian	Guardia® Access catheter (-80ºC)	Negative controls: blank controls	Cell lysis and DNA purification (Agencourt AMPure® XP Reagent); Illumina MiSeq® (V3-4-6)	*Lactobacillus*, *Gardnerella*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Atopobium*, *Delftia*, *Pelomonas*, *Veillonella*, *Escherichia coli/Shigella*	PRJNA634237 available at NCBI BioProject	Biodiversity was greater in the endometrial microbiome than in the vagina Biodiversity was significantly greater after COS when compared with pre-COS in the same patients Contaminants: *Sphingomonas*, *Arthrobacter*, *Renibacterium*
Chen *et al*. 2020	Control group (infertility, leiomyoma, ovarian borderline tumour or teratoma) (N=2); avg 36.07y; Asian	Vacuum suck tube (-80ºC)	Cervical canal was sterilised with iodine	CTAB/SDS method; Illumina HiSeq 2500® (V3-4)	*Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Atopobium*	http://atm.amegroups.com/article/view/5402/38885 (available from June 2021)	The outcome of the PCR from uterine samples was barely satisfactory No valid evidence of uterine sterility
Lu *et al*. 2020	Endometrial cancer (N=25); 18–75y; Benign condition (N=25); Asian	Endometrial biopsy at hysterectomy (-80ºC)	Negative control: blank buffer control	SDS method; Illumina HiSeq® (V3-4)	*Rhodococcus*, *Phyllobacterium*, *Sphingomonas*, *Bacteroides*, *Bifidobacterium*	Available from the corresponding author on reasonable request	Significant differences in α- and β-diversity between cancer and benign condition groups Increased abundance of *Micrococcus* in endometrial cancer group

The systematic literature search was performed up to 5 January 2021 using PubMed, Web of Science and Cochrane databases, and was registered with the PROSPERO (registration number: CRD42020161218). Keywords "endometrium", "endometrial fluid", "uterus", "uterine fluid" were paired with terms "microbiome", "microbiota", "microbes", "microorganisms". A total of 15 292 studies were identified and following critical selection 119 manuscripts of endometrial microbiome studies were selected. Next, after careful abstract and full-text evaluation, 34 manuscripts remained eligible.

Avg: average; CE: chronic endometritis; COS: controlled ovarian stimulation; ENA: European Nucleotide Archive; EP: endometrial polyps; ET: embryo transfer; IVF: *in vitro* fertilisation; NR: non reported; RIF: recurrent implantation failure; RPL: recurrent pregnancy loss; spp., species; SRA: Sequence Read Archive; y: years old.

*The first metagenomics study performed in human endometrium.

The next important aspect to consider is the reproducibility of the studies. Similar microbiome studies can produce different results, and without detailed documentation of the study population, sample type, collection method, data processing, and analysis workflow, i.e., the creation of metadata, it is difficult to reproduce the data (Jurburg *et al.*, 2020). The recorded metadata would ensure that as much variability as possible is accounted for, and it should be made publicly available together with the raw data. However, metadata deposited in repositories (e.g., sequence read archive, SRA) are not standardised, creating difficulty for sample reanalysis (Kasmanas *et al.*, 2020). We also recommend the researchers to follow the Genomic Standards Consortium minimum information standards for marker genes (MIMARKS) and metagenome (i.e., sequencing of microbiome) analyses (MIMS) (Yilmaz *et al.*, 2011; Quiñones *et al.*, 2020), or other initiatives such as BioProject and BioSample project, which outline the minimum necessary information about a metagenomic sample (Kasmanas *et al.*, 2020).

CONSIDERATIONS ON STUDY POPULATION

Accurate selection of patients and controls is the next crucial step in planning a quality research project, where it is important to consider endometrial microbiome dynamics and factors that could influence the uterine environment (see review for factors by (Molina *et al.*, 2020)). It is still debatable whether endometrial microbiome changes throughout the menstrual cycle or not (Altmäe, 2018). Some studies have detected that endometrial microbiome is highly stable throughout the menstrual cycle (Khan *et al.*, 2016; Moreno *et al.*, 2016; Kyono *et al.*, 2018), while other groups have found significant differences in the composition of uterine microbes between proliferative, secretory, and menstrual phases (Chen *et al.*, 2017; Pelzer *et al.*, 2018; Kadogami *et al.*, 2020; Sola-Leyva *et al.*,

2021). Clearly, more investigation into identification of the uterine "baseline" microbial continuum along the menstrual cycle is warranted.

The selection of proper control/patient groups depends on the specific research question; nevertheless, a detailed metadata information is required for all participants in order to have comparable study groups and to control for possible confounding factors. Furthermore, nullipara, para, and multipara women should be distinguished, as it has been shown that pregnancy and childbirth can influence microbial composition of the female reproductive tract (Koedooder *et al.*, 2019).

CONSIDERATIONS ON ENDOMETRIAL SAMPLING AND STORAGE

The biggest concern regarding endometrial sampling for microbiome analysis is the extremely high risk of contamination from lower genital tract. Sampling during invasive surgical procedures, such as hysterectomy and laparoscopy, avoids contamination with microbes from the vagina and/or cervix, however, these samples are usually obtained from women with an existing medical condition and from peri- and post-menopausal women, thus the results are not necessarily applicable to healthy reproductive age women.

In addition to the conventional endometrial sampling devices such as Pipelle and swabs, a few uterine sampling methods have been adapted for microbiome studies in order to minimise the risk of contamination: double-sheathed embryo transfer catheter (Franasiak *et al.*, 2016), intrauterine insemination catheter (Kyono *et al.*, 2018), and a transcervical sheathed brush device (Verstraelen *et al.*, 2016). Like with every step in a study protocol, effort should be made to collect samples in a standardised manner throughout the study for minimising unintentional variability. A recent report compared microbial colonisation in endometrial fluid and endometrial tissue samples that were collected simultaneously

and concluded that the microbiome composition in the fluid does not fully reflect that in the tissue (Liu *et al.*, 2018).

Once collected, storage and transport of the samples under bacteriostatic conditions are critical, as sample handling and treatment can influence the composition of a microbial community (Karstens *et al.*, 2018; Fricker *et al.*, 2019). A quality microbiome study should also record sampling and storing steps in metadata collection. Direct freezing of the collected samples at –80°C is considered as the reference method in microbiome studies (Koedooder *et al.*, 2019). A comparative study on gut showed that there were no considerable differences between 16S rRNA gene sequencing outcomes from fresh samples, samples frozen at –80°C, or samples that were snap-frozen on dry ice and then at −80°C (Fouhy *et al.*, 2015). Of note, snap-freezing reduces ice crystal formation in the sample compared with direct freezing at −80°C, thus retaining a better cell integrity (Fouhy *et al.*, 2015).

In a clinical setting, however, –80°C freezer is often not available and different storage buffers/media could serve as alternatives that can stabilise nucleic acids at 4°C or even at room temperature for days (Karstens *et al.*, 2018). Certain concentrated buffered solutions such as RNAlater, PSP stabilisation buffer, Allprotect Tissue Reagent, or medium that stabilises microbes and maintains RNA and DNA integrity of bacteria and viruses (e.g., stabilisers by COPAN Diagnositics, DNA Genotek, Norgen Biotek and others) are available in market. Microbial community analysis from samples stored in RNAlater, however, has shown the least similar bacterial communities compared to fresh samples and the samples stored at −80°C (Pollock *et al.*, 2018). Another study compared the use of six storage solutions (Norgen, OMNI DNA Genotek, RNAlater, CURNA, HEMA, and Shield) and found that samples collected in Norgen and OMNI showed the least shift in

community composition relative to −80°C standards compared with other storage media (Chen *et al.*, 2019).

In short, different endometrial microbiome studies using different sampling and storage methods (**Table 1**) are hardly comparable and the field is yearning for meticulously designed large cohort studies specifically adapted to the low microbial biomass niche.

DETECTION OF MICROBIOME

Technical variation among experimental methods ranging from nucleic acid extraction to sequencing is high (Fricker *et al.*, 2019). Commercial kits for microbial nucleic acid isolation show different efficacy in lysing specific microbes, which can impact the observed microbial patterns (Pollock *et al.*, 2018). Different methods for detecting microbial communities including marker gene (prokaryotic 16S rRNA and fungal ITS), metagenome, and metatranscriptome sequencing (see **Figure 1** for illustration of detection techniques) can also give rise to diverse results (Knight *et al.*, 2018). All these detection methods have their strengths and limitations (see **Table 2**), and the choice of method would depend on the study question, design, and budget.

Marker gene analysis

The most commonly used marker gene is 16S rRNA gene (**Figure 1**). Despite 16S rRNA gene being specific to bacteria and archaea, additional marker genes for eukaryotic microorganisms, like fungi, exist 18S rRNA gene, the 28S rRNA gene, and the ITS (Fricker *et al.*, 2019).

Marker gene amplification and sequencing is well-tested, fast, and cost-effective for attaining a low-resolution information of microbial communities. This method is especially suitable for samples contaminated by host DNA (Knight *et al.*, 2018) such as

tissues and low microbial biomass samples as is the endometrium. However, different methodological considerations should be considered when opting for this detection method (**Table 2**). In the endometrial studies, the most frequently used 16S rRNA hyper-variable region is V4 (**Table 1**). A study comparing V1-2 and V3-4 hyper-variable regions when analysing vaginal microbiome concluded that V3-4 identifies more taxa and displays higher diversity than the V1-2 region, since V1-2 region failed to identify some important species harbouring the female lower genital tract (Graspeuntner *et al.*, 2018).

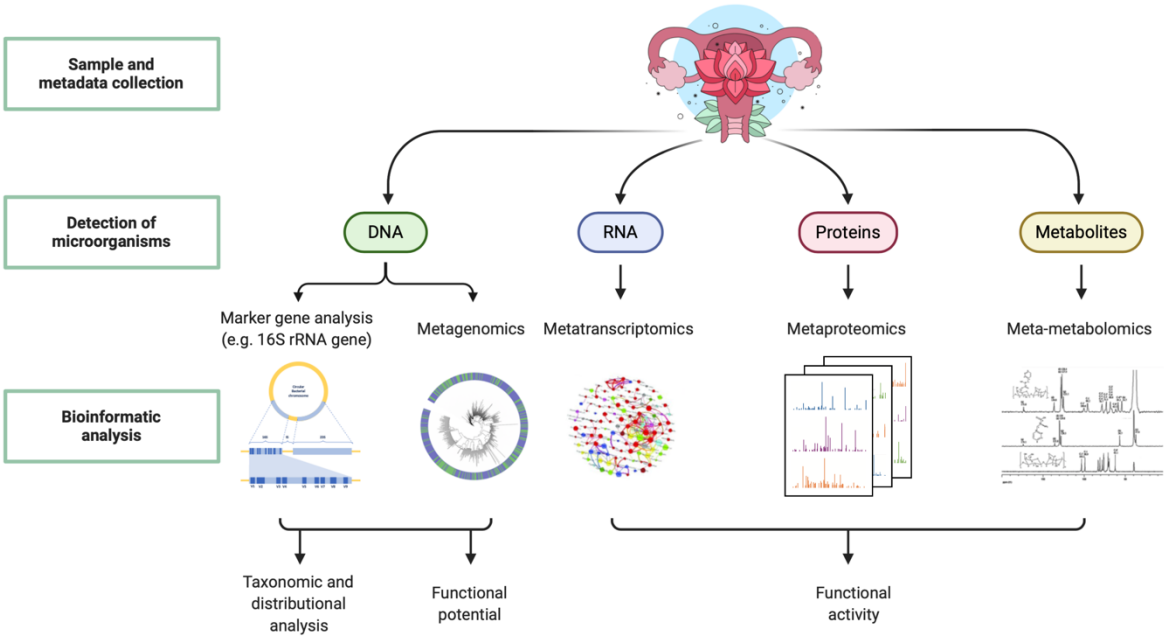

Figure 1. Different techniques for studying human microbiome: marker gene analysis, whole genome sequencing, metatranscriptomic sequencing, metaproteome, and meta-metabolome analyses. Marker gene analysis targets a specific sequence of a gene in order to identify microbial phylogenies in a sample. The most commonly used marker gene is 16S rRNA gene. The 16S and 23S rRNA genes are highlighted together with the ITS region. V1–9 marks the hyper-variable regions, with the conserved regions between them. Whole metagenome sequencing analysis (metagenomics) consists of sequencing of all genes and genomes in a microbial community, which does not depend on amplifying and sequencing specific taxonomically informative genes. Metagenome represents the genetic/functional potential of the microbes, not the actual activity of the community. To analyse the functional activity (metatranscriptomics), the isolation of messenger RNA (mRNA) is necessary. It is also possible to characterise the function of microbial communities through the analysis of proteins expressed by microorganisms (metaproteomics), which offers better knowledge of the intricate host–microbiome interactions. Generally, metatranscriptome and/or metaproteome studies are complemented by identification and quantification of chemical compounds present in the sample (meta-metabolomics) in order to correlate gene expression to the metabolite profiles.

Despite several limitations in marker gene analysis (**Table 2**), this approach is the most commonly used and preferred for the low microbial biomass microbiome studies today (Knight *et al.*, 2018; Liu *et al.*, 2020). In fact, all endometrial microbiome studies conducted so far are based on the marker gene analysis, except for the preliminary analysis of the five endometrial samples where the whole metagenome analysis approach has been applied (Li *et al.*, 2018; Garcia-Grau *et al.*, 2019; Moreno *et al.*, 2020) (**Table 1**).

Whole metagenome analysis

This approach yields more detailed genomic information and taxonomic resolution than the marker gene sequencing method, capturing all microbial genomes present in the sample, including viral and eukaryotic DNA (Liu *et al.*, 2020; Qian *et al.*, 2020) (**Figure 1**; strengths and limitations in **Table 2**). As the metagenomics field matures, the current limitations (especially the annotation steps) will continue to improve.

The preliminary results in metagenome analysis have identified different taxonomies of bacteria, archaea, fungi, and viruses within the endometrial samples (Li *et al.*, 2018); however, greater depth of sequencing, better reference database and more sample material are required in order to meet the full coverage scale. Undoubtedly, assessing microorganisms other than bacteria in endometrial/uterine health and disease is an important future research area.

Metatranscriptome analysis

Unlike marker gene and metagenomic sequencing, where DNA sequences in a sample are analysed regardless of the cell viability or activity, metatranscriptomics uses RNA sequencing to profile transcripts of microorganisms to provide information of gene expression and the functional activity of the microbiome (Knight *et al.*, 2018). One of the biggest challenges for metatranscriptome analysis is the identification and removal of the

host ribosomal sequences (>95% of extracted bacterial RNA is non-coding rRNA and thus not informative for active expression) in order to enrich the microbiome transcripts (see **Table 2** for pros and cons).

Recently, the first endometrial metatranscriptomic analysis revealed that endometria of healthy women harbour more than 5000 functionally active microorganisms (Sola-Leyva *et al.*, 2021). This study also demonstrated that several bacteria, viruses, and archaea are differentially regulated along the menstrual cycle and these cyclical changes could be associated with metabolic activity in the host-microbiota crosstalk during receptive phase endometrium (Sola-Leyva *et al.*, 2021).

Table 2. High-throughput techniques to analyse the endometrial microbiome

Technique	Function	Advantages	Limitations
Marker gene analysis (e.g., 16S rRNA gene sequencing)	Amplification and sequencing of the 16S rRNA gene - a specific hypervariable region or a set of contiguous hypervariable regions	Relatively rapid, simple, and affordable technique Classifies microorganisms to taxonomic classification Correlates well with genomic content Applicable to many sample types and study designs Adaptable to low biomass and highly host-contaminated samples (e.g., endometrial tissue) Requires lower quantity of DNA input (≈50 ng)	Requires a priori knowledge of microbial community Limited to explore microbial diversity Taxonomical resolution typically limited to genera level Some bacteria contain several copies of 16S rRNA Indistinguishable species (some species have <0.5% sequence divergence) Limited identification of variant strains (does not contemplate horizontal gene transfer) Different hypervariable regions yield different results, making comparisons between studies challenging Technical bias where some species are amplified more than others Limited functional analyses No information about bacteria viability Chimeric errors DNA sequencing errors Contaminant errors

Whole metagenome (shotgun) analysis	Breaks the full DNA content into small constant fragments that can be sequenced and aligned to a reference catalogue	Captures the full genetic information (bacteria, archaea, viruses, eukaryotic microorganisms), including that of unknown species Does not require knowledge of microbial community No PCR-related biases Allows direct quantification of microbial gene abundance and microbial genome reconstruction Predicts the potential function of the microbiota based on their genomes Enables a more specific taxonomic resolution at the species level Identification of strains Can estimate *in situ* growth rates for target organisms	More expensive and laborious Requires complex bioinformatics and biostatistics pipelines Assembly artefacts Requires higher quality and higher amounts of DNA (>1 μg) Requires removal of host and ribosomal sequences Just gives information about the potential functionality Huge amounts of data produced (specific information is diluted) Lacking universal reference databases Difficult quantification of a particular organism in a sample Viruses and plasmids are not typically well annotated by default pipelines No information about bacteria viability
Metatranscriptome analysis (meta-RNAseq)	Sequencing of the transcribed RNA in a microbial community after undergoing samples to microbial rRNA and host nucleic acid removal	Directly evaluates microbial functional activity Informs about the viability/dormancy of microorganisms Represents a link between the metagenome and community phenotype Quantifies gene expression level, detecting genes that are differentially expressed/regulated Captures dynamic intra-individual variations	Expensive and laborious technique Difficult to maintain the resemblance of the *in situ* expression levels Abundance of the microorganisms and their gene expression levels can exhibit variation in transcript levels Methods used for single-organism transcriptomics cannot be applied Host RNA and microbial rRNA must be removed (ribodepletion, mRNA enrichment) Requires creating a complex and proper reference library Requires paired DNA sequencing to decouple transcription rates from bacterial abundance changes Different strains, or even different species, can maintain a similar functional profile Data are biased towards organisms with high transcription rates
Metaproteome analysis	Shears the peptides into small fragments	Informs about taxonomic distribution	Time-consuming methodology

	that undergo a mass spectrometry and the resulting amino acid sequence is compared to a reference databases	Characterises all gene translation products (sequential variants resulting from splicing processes) Provides insights on post translational modification (identification of alterations in structure) Informs about the viability/dormancy of microorganisms Analyses interactions between species present in a community Informs about protein stability and localization Identifies habitat-specific functions covered by the community	High sensitivity to sample impurities Subject to inefficient chemical labelling leading to compromised biological coverage Does not provide information on protein abundance Requires an environment specific protein database Requires high amount of protein biomass from samples
Meta-metabolome analysis	Examines metabolites through liquid/gas chromatography, mass spectrometry and nuclear magnetic resonance and metabolomic data is compared to spectral databases	Analyses of numerous metabolites in a given sample High predictive capacity for phenotype Resolution of microbial metabolic products/signalling molecules	High cost Rather emerging (currently not possible to translate all the data produced into a meaningful biological context) Databases may contain low quality reference mass spectrometry spectra Enormous chemical diversity hinders molecular identification Rapid turnover of metabolites Difficult to determine whether a metabolite was produced by the host or by the microbiome

rRNA: ribosomal RNA; mRNA: messenger RNA

COMPUTATIONAL CONSIDERATIONS

Microbiome data analysis methods are quickly advancing, and computational considerations and challenges are evolving alongside them. Several platforms and algorithms exist, with a major issue being their ability to guarantee proper alignment and fidelity of the data while successfully subtracting contamination and background noise (Callahan *et al.*, 2017). The recommendations regarding differential abundance testing using ASVs over operational taxonomic units (OTUs) have advanced especially fast

(Knight *et al.*, 2018). ASV methods deduce the biological sequences in the sample before amplification and sequencing errors and distinguish sequence variants differing by as little as one nucleotide (Callahan *et al.*, 2017). These new ASV methods are expected to replace OTUs as the unit of analysis.

Many pipelines are available today to process and analyse 16S rRNA gene sequencing data, where QIIME, QIIME 2, UPARSE, USEARCH, VSEARCH, and Mothur are the most commonly used (Liu *et al.*, 2020). An additional factor that could influence the resolution of the analysis is database selection for taxon identification (Knight *et al.*, 2018). Different curated databases of 16S rRNA gene sequences exist, such as Greengenes, SILVA, NCBI, and Ribosomal Database Project (RDP) classifier (Karstens *et al.*, 2018). The last three contain taxonomic information for the domains of Bacteria, Archaea, and Eukarya, while Greengenes is dedicated to Bacteria and Archaea (Balvočiūtė and Huson, 2017). SILVA is the most updated and largest database, allowing identification at the species level in a good number of taxa (Balvočiūtė and Huson, 2017), and is commonly used for 16S rRNA gene studies. However, one should consider using the NCBI database in studies that perform both targeted 16S rRNA gene sequencing and shotgun sequencing (Balvočiūtė and Huson, 2017).

For further reading about bioinformatics tools and how they can be applied for targeted sequencing, metagenomic, and metatranscriptomic analyses please see previous comprehensive reviews (Knight *et al.*, 2018; Liu *et al.*, 2020; Qian *et al.*, 2020). In the last years, several initiatives have arisen to harmonise metadata from multiple human metagenomic studies in a single standardised database in order to readily filter the data for samples of interest, including standardised attributes describing host characteristics, sampling site and/or condition information. Specifically, HumanMetagenomeDB aims to explore possibilities of human metagenomes from different body sites (e.g., gut, skin,

vagina, however, not including uterus) in a user-friendly interface, leading to accurate meta-analyses (Kasmanas *et al.*, 2020). Furthermore, Vaginal Microbiome Consortium is creating a specific database for vaginal samples (http://vmc.vcu.edu/vahmp) and similar activities are strongly encouraged for endometrial samples.

COMBATING CONTAMINATION

It is clear that low biomass microbial niches such as uterus are sensitive to contamination and data misinterpretation. Two fundamental types of contamination can arise in microbiome studies, contaminant DNA and cross-contamination (Eisenhofer *et al.*, 2019). To start with, the contaminant DNA can originate from the technique of endometrial sampling, but also from laboratory environment, plastic consumables, researchers, and reagents (Eisenhofer *et al.*, 2019). Many groups have demonstrated that laboratory stocks used in processing and analysing DNA are contaminated with bacterial DNA—jointly termed as "kitome" (unsurprising as many enzymes/compounds in them are derived from bacteria) (Kim *et al.*, 2017). The kitome varies between kits and can even be different between different lots of the same kit (Salter *et al.*, 2014; Glassing *et al.*, 2016). Thus, it is strongly recommended to process all samples side by side using the same batches of reagents. In fact, over 100 common contaminant taxa have been detected in DNA extraction blank controls and no-template controls across many studies (**Table 3**). Cross-contamination is another challenge during microbiome sample processing and can originate from other samples and sequencing runs (Eisenhofer *et al.*, 2019). It has been demonstrated that lower levels of microbial DNA within low biomass samples enable contaminant DNA and cross-contamination to outcompete and dominate the biological signal within the sample (Salter *et al.*, 2014; Glassing *et al.*, 2016).

Regardless of the study approach, appropriate controls are vital and mandatory for microbiome studies. Low biomass environments, such as the endometrium, require controls that have gone through the entire sampling process in order to fully detect contaminants and to distinguish the low-abundance microorganisms that truly originate from the sampling site (Salter *et al.*, 2014; Benner *et al.*, 2018; Qian *et al.*, 2020). Three types of negative controls are recommended to adequately detect contaminants: 1) sampling blank control; 2) DNA extraction blank control; and 3) no-template amplification control (Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019). As for the positive controls, two types are recommended to use for determining the limit of detection and to evaluate the effect of cross-contamination during the sample processing: 1) DNA extraction positive control and 2) positive amplification control (Eisenhofer *et al.*, 2019).

Table 3. List of contaminant genera most commonly identified in human microbiome studies

Phylum	List of contaminant genera
Proteobacteria	Alpha-proteobacteria: *Afipia*[a], *Aquabacterium*[b], *Asticcacaulis*[b], *Aurantimonas*[a], *Beijerinckia*[b], *Bosea*[b], *Bradyrhizobium*[a], *Brevundimonas*[a], *Candidatus*[a], *Caulobacter*[a], *Chelatococcus*[a], *Craurococcus*[b], *Devosia*[a,b], *Gluconobacter*[a], *Hoeflea*[b], *Mesorhizobium*[a], *Methylopila*[a], *Methylobacterium*[a,b], *Novosphingobium*[a,b], *Ochrobactrum*[a], *Pannonibacter*[a], *Paracoccus*[b], *Pedomicrobium*[b], *Phenylobacterium*[a], *Phyllobacterium*[a], *Pseudochrobactrum*[a], *Rhizobium*[b], *Rhizomicrobium*[a], *Roseomonas*[a], *Rubellimicrobium*[a], *Ruegeria*[a], *Sphingobium*[a,b], *Sphingomonas*[a,b], *Sphingopyxis*[b], *Telmatospirillum*[a]
	Beta-proteobacteria: *Achromobacter*[a], *Acidovorax*[a], *Alicycliphilus*[a], *Azoarcus*[b], *Azospira*[b], *Burkholderia*[a], *Comamonas*[a,b], *Cupriavidus*[a,b], *Curvibacter*[a], *Delftia*[b], *Duganella*[b], *Herbaspirillum*[a], *Janthinobacterium*[a], *Kingella*[a], *Leptothrix*[a], *Limnobacter*[b], *Limnohabitans*[a,b], *Massilia*[a], *Methylophilus*[b], *Methyloversatilis*[b], *Neisseria*[a], *Oxalobacter*[a], *Parasutterella*[a], *Pelomonas*[a], *Polaromonas*[b], *Ralstonia*[a,b], *Roseateles*[a], *Schlegelella*[a], *Sulfuritalea*[a], *Tepidimonas*[a], *Undibacterium*[b], *Variovorax*[a], *Vogesella*[a]
	Gamma-proteobacteria: *Acinetobacter*[a,b], *Alcanivorax*[a], *Cardiobacterium*[a], *Citrobacter*[a], *Dokdonella*[a], *Dyella*[a], *Enhydrobacter*[a], *Enterobacter*[a], *Escherichia*[a], *Haemophilus*[a], *Halomonas*[a], *Klebsiella*[a], *Lysobacter*[a], *Nevskia*[b], *Pseudomonas*[a,b], *Pseudoxanthomonas*[b], *Psychrobacter*[b], *Serratia*[a], *Shewanella*[a], *Stenotrophomonas*[a], *Xanthomonas*[b], *Yersinia*[a]
Actinobacteria	*Actinomyces*[a], *Aeromicrobium*[b], *Agrococcus*[a], *Arthrobacter*[a], *Atopobium*[a], *Beutenbergia*[b], *Bifidobacterium*[a], *Blastococcus*[a], *Brevibacterium*[a], *Collinsella*[a], *Corynebacterium*[a], *Curtobacterium*[b], *Dermacoccus*[a], *Dietzia*[a], *Eggerthella*[a], *Geodermatophilus*[b], *Gordonia*[a], *Janibacter*[b], *Kocuria*[b], *Microbacterium*[a], *Micrococcus*[a,b], *Microlunatus*[a], *Patulibacter*[b], *Pilimelia*[a], *Propionibacterium*[a], *Pseudoclavibacter*[a], *Renibacterium*[a], *Rhodococcus*[a], *Rothia*[a], *Slackia*[a], *Tsukamurella*[b], *Zimmermannella*[a]

Firmicutes	*Abiotrophia*[a], *Alicyclobacillus*[a], *Anaerococcus*[a], *Anaerotruncus*[a], *Bacillus*[a], *Blautia*[a], *Brevibacillus*[a], *Brochothrix*[b], *Catenibacterium*[a], *Christensenella*[a], *Clostridium*[a], *Coprococcus*[a], *Dialister*[a], *Dorea*[a], *Enterococcus*[a], *Erysipelatoclostridium*[a], *Eubacterium*[a], *Exiguobacterium*[a], *Facklamia*[a], *Faecalibacterium*[a], *Fastidiosipila*[a], *Finegoldia*[a], *Flavonifractor*[a], *Gemella*[a], *Geobacillus*[a], *Granulicatella*[a], *Halocella*[a], *Intestinibacter*[a], *Johnsonella*[a], *Lachnoanaerobaculum*[a], *Lachnoclostridium*[a], *Lachnospira*[a], *Lactobacillus*[a], *Lactococcus*[a], *Leuconostoc*[a], *Megasphaera*[a], *Moryella*[a], *Oscillospira*[a], *Paenibacillus*[a], *Papillibacter*[a], *Parvimonas*[a], *Peptococcus*[a], *Peptoniphilus*[a], *Pseudobutyrivibrio*[a], *Pseudoflavonifractor*[a], *Quinella*[a], *Roseburia*[a], *Ruminiclostridium*[a], *Ruminococcus*[a], *Sanguibacter*[a], *Selenomonas*[a], *Solobacterium*[a], *Staphylococcus*[a], *Streptococcus*[a], *Thermicanus*[a], *Trichococcus*[a], *Tumebacillus*[a], *Turicibacter*[a], *Tyzzerella*[a], *Veillonella*[a]
Bacteroidetes	*Alistipes*[a], *Bacteroides*[a], *Capnocytophaga*[a], *Chryseobacterium*[a], *Cloacibacterium*[a], *Dyadobacter*[b], *Elizabethkingia*[a], *Filimonas*[a], *Flavobacterium*[a], *Fluviicola*[a], *Hydrotalea*[a], *Hymenobacter*[a], *Niastella*[b], *Olivibacter*[b], *Parabacteroides*[a], *Pedobacter*[a], *Porphyromonas*[a], *Prevotella*[a], *Sediminibacterium*[a], *Sphingobacterium*[a], *Wautersiella*[a], *Xylanibacter*[a]
Deinococcus-Thermus	*Deinococcus*[a], *Meiothermus*[a]
Fusobacteria	*Fusobacterium*[a], *Leptotrichia*[a]
Spirochaetes	*Leptospira*[a]
Acidobacteria	Predominantly unclassified *Acidobacteria* Gp2 organisms[a]

The listed genera were detected in sequenced negative controls, consisting of DNA extraction blanks from different DNA extraction kits, and no-template PCR amplification controls from PCR kits (Salter *et al.*, 2014; Glassing *et al.*, 2016; Kyono *et al.*, 2018; Eisenhofer *et al.*, 2019; Hashimoto and Kyono, 2019; Kitaya *et al.*, 2019; Weyrich *et al.*, 2019; Winters *et al.*, 2019; Carosso *et al.*, 2020; Kadogami *et al.*, 2020).
Source of contamination: [a] Extraction blank control; [b] No-template amplification control

A single known microbial isolate or a mock community (known mixture of microbial species or a synthetic non-biological DNA) could serve as positive controls (Kim *et al.*, 2017; Karstens *et al.*, 2018; Pollock *et al.*, 2018).

The contaminant taxa must be addressed in the final analysis and interpretation of the results. As biomass decreases, the influence of contaminating sequences becomes more evident (Weyrich *et al.*, 2019; O'Callaghan *et al.*, 2020). Three different strategies for assessing the impact of contamination in the microbiome datasets are available: 1) comparison of controls to biological samples; 2) subtracting contaminants from biological samples (e.g., Decontam, microDecon); and 3) using predictive modelling for detecting contaminants (e.g., Sourcetraker) (see (Eisenhofer *et al.*, 2019) for further reading).

To sum up, contamination has been and will be one of the biggest combats in analysing endometrial microbes and it is clearly challenging to control for all possible contaminants.

Nevertheless, we should aim transparency in presenting study results, provide raw data together with metadata (also for negative/positive controls) and address the study limitations adequately.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our understanding of the endometrial microbiome is still in its infancy and myriads of questions remain to be addressed. Whether the microbiome detected in the endometrium is merely the result of contamination and an artefact of the study design, and/or whether the microbes in the endometrium are tourists, invaders, or residents need to be established by large and well-designed, well-controlled, and well-conducted studies.

Based on the currently available studies' designs, no conclusive information on microbial detection and distribution of a "core uterine microbiome" is available. Before the presence of a microbiome can be attributed to a disease risk and pathogenesis, and before any treatment options for bacterial "dysbiosis" are offered for patients (Haahr *et al.*, 2020), acquisition and development of the "normal" microbiome must be well established (Chu *et al.*, 2019). To that point, the role of the endometrial microbiome and/or microbiota is not yet established, and the nature of the relationship between the endometrial microbiome and the function of the female reproductive tract remains an open issue. Furthermore, a sole presence of a microbial DNA sequence does not equate to the presence of a live microorganism, and the question of the viability of microbes detected by the NGS requires further research (Benner *et al.*, 2018; Altmäe *et al.*, 2019). DNA sequences can represent microbial breakdown products (e.g., DNA from dead microbes) or background DNA contamination (Kim *et al.*, 2017), thus detection of a nucleotide sequence of a bacteria is not the same as the identification of a living microorganism;

DNA can be used to characterise a microbiome but not to establish its existence (Salter *et al.*, 2014; Glassing *et al.*, 2016; Kim *et al.*, 2017).

Although the functional activity of a given community could be predicted through analysing the microbial gene content (e.g., PICRUSt), future analyses of the expressed proteins within a microbial community (i.e., metaproteome) and characterisation of metabolites generated by microbes (i.e., meta-metabolome) (see **Figure 1** for methods) would further unravel the complexity of microbial functions in the endometrium.

As the endometrial microbiome field continues to rapidly expand, there is a great need for clear, concise, and well-tested protocols starting with study planning and sample collection up to bioinformatic analyses. Three minimal standard requirements that researchers should follow for human microbiome studies have been proposed (Greathouse *et al.*, 2019): 1) describe in detail nucleic acid extraction methods employed in the study so that all extraction procedures can be reproduced; 2) include and describe negative and positive controls, and report contamination in blank samples; and 3) follow the same study protocol along entire project. It is unlikely that the entire field will agree to the exact protocols and workflows, but scientists, reviewers, and journals should aim to improve our current protocols and reporting criteria to ensure reliability and consistency between studies. With this review, we hope to provide the much-needed guidance, recommendations, and best practices for designing and conducting endometrial microbiome studies and to draw attention to the weak points that could be improved or avoided in order to ensure that quality microbiome research in endometrial microbiome field is undertaken (see **Table 4** for the specific recommendations). This methodological review provides an initial framework to help to establish community guidelines and maximise the potential of this emerging and highly interdisciplinary field of research.

Table 4. The current good practice recommendations for performing endometrial microbiome studies

Study design	• Set study hypothesis • Define study type (e.g., case-control, longitudinal)
Study population	• Calculate sample size and power (Dirichlet Multinomial, PERMANOVA, random forest analyses) • Specify participant inclusion/exclusion criteria • Select and define control group • Define confounding factors (e.g., age, medical history, medication, ethnicity, BMI, diet, sexual habits, pregnancy) • Create metadata (detailed information of participants, sample collection and storage, microbiome detection and analysis)
Endometrial sampling	• Record cycle day for sampling as microbiome can change throughout the cycle • Using adapted sampling methods (e.g., double-sheathed catheter, sheathed brush device) – currently the best method as reduces cervical/vaginal bacterial contamination risk and minimally invasive • Using surgical procedures (hysterectomy, laparoscopy) avoids transcervical/vaginal bacterial contamination, but limitation with obtaining samples from healthy controls • Using transcervical sampling (e.g., Pipelle) – the most commonly used technique, but high bacterial contamination risk from cervix/vagina • Include negative controls (e.g., from gloves, unused surgical tools, swab of air) - allows adequate monitoring of contaminants throughout sample collection and handling
Sample storage	• Snap-freezing and direct freezing of samples at –80°C are the reference methods (alternative is to use storage media that stabilises nucleic acids at higher temperatures) • Avoid repeated freezing-thawing • Record sampling and storage protocols in metadata
Detection of microbiome	• Adequate cell lysis and host DNA elimination are crucial in extracting microbial DNA – one of the recommended kits today for low microbial biomass site is QIAamp DNA Microbiome Kit (Bjerre *et al.*, 2019; Heravi *et al.*, 2020) • Include positive controls (e.g., bacterial mock community) • Include negative controls (e.g., blank sample) • Report in detail DNA extraction method and controls • Assess microbial DNA quality and quantity (e.g., spectrophotometry) • 16S rRNA marker gene analysis is the recommended method for low microbial biomass site analysis as is endometrium • Sequencing 16S rRNA gene hypervariable regions 3-4 is the most commonly used and has been shown to be more sensitive over V1-2 • Utilise the same protocols throughout the study
Microbiome data analysis	• Process and analyse sequencing data with updated methods (e.g., QIIME2, Mothur) • Use ASVs over OTUs

	• SILVA is the most updated and largest database for alignment – commonly used for 16S rRNA studies • Remove contaminant sequences *in silico* (e.g., Decontam, microDecon, Sourcetracker) • Predict microbial functional activity (PICRUSt, Tax4Fun, BugBase) • Report in detail the analysis steps
Data validation	• Quantify detected bacteria (e.g., flow-cytometry, qPCR, spiking of exogenous bacteria into crude samples) • Validate in separate cohort
Data presentation	• Deposit raw data and metadata to public database (e.g., SRA repository, ENA) • Characterise and report contaminant microbes • Publish in detail the study protocols • Address limitations/strengths of the study

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GENERAL DISCUSSION

In the male population, deeper knowledge of the microbial mechanisms underlying health and disease are warranted. This Doctoral Thesis sought to increase our current knowledge of the microbiome within the male reproductive tract, focussing specifically on its composition and origins. Through the four studies conducted, it became apparent that this field is full of limitations, inconsistencies, and complexities, requiring further investigation. First, we studied microbes in the human testicular samples by analysing maturing spermatozoa using 16S rRNA gene sequencing (**Study I**). Second, we explored the potential contribution of the upper reproductive tract together with the urinary microbiome on the seminal microbial composition by comparing semen and urine samples before and after the sterilisation procedure via vasectomy (**Study II**). Third, we provided a systematic overview of the shared microbial compositions among genital tracts within the couple (**Study III**). And lastly, we highlighted the methodological considerations and proposed good practice recommendations for low biomass microbiome studies in reproductive medicine, using endometrium as the low microbial biomass site (**Study IV**).

The seminal microbiome hosts an intricate blend of various bacterial genera, encompassing both aerobic and anaerobic microbes, and even potential pathogens. We demonstrate a plausible contribution of the upper reproductive tract into the seminal microbial composition by revealing the presence of microorganisms in the maturing spermatozoa from human testes (**Study I**). Ten sperm-specific genera were identified in the testicular samples *Blautia*, *Cellulosibacter*, *Clostridium* XIVa, *Clostridium* XIVb, *Clostridium* XVIII, *Collinsella*, *Prevotella*, *Prolixibacter*, *Robinsoniella*, and *Wandonia*. Among them, *Prevotella*, *Blautia*, and *Clostridium* are commonly detected in the seminal microbiome studies (Altmäe *et al.*, 2019). Despite different microbial genera detected, it

is a low microbial biomass site and our samples presented abundant contamination. As sampling of testicular samples involves an invasive method and is not performed on healthy men, it is difficult to analyse this niche in the disease-free population. Meaning that the few studies performed to date should be considered as preliminary and require further confirmation. Next, we compared paired semen samples before and after the vasectomy and found that the overall relative abundance of the identified genera remained similar between these two time points (**Study II**). However, significant differences in the microbial richness (i.e., α-diversity) and microbial dissimilarity (i.e., β-diversity) were observed, which support the hypothesis of the paracrine contribution of upstream anatomic locations on seminal microbial composition. Despite this novel evidence of the origin of the seminal microbiome, some genera that seem other than testicular origin were found. To address this, we compared paired urine and semen samples and revealed that the urine is much more diverse than semen, which may translate into a source of microorganisms via urethral sharing (**Study II**). We also found that urine and semen share up to half of their microbiomes (e.g., *Lactobacillus*, *Veillonella*, and *Finegoldia*, among others). A number of the described microbes in the urine and semen can form biofilms which could allow them to persist in the genitourinary tract, colonising both the urinary and reproductive systems. Indeed, *Corynebacterium* detected in the semen and urine has been shown to display biofilm-forming capabilities (Türk *et al.*, 2014), providing an advantage of surviving and growing in these niches.

Further, it has been shown that sexual debut enriches the seminal microbiome with diversity. Similarities in the microbial composition between the semen and vaginal samples within couples suggest an active interplay of microbial communities during sexual intercourse, having implications for reproductive health, which was systematically reviewed and summarised (**Study III**). The exchange of microorganisms via sexual

activities between partners can have both beneficial and detrimental effects on the health of the couple. Indeed, successful IVF outcomes has been correlated with increased mean proportions of *Lactobacillus.* However, the influence of *Lactobacillus* on sperm quality needs to be further investigated, as direct adherence to spermatozoa has been shown to reduce sperm motility (Wang *et al.*, 2020). In a certain way, a *Lactobacillus*-based probiotic treatment could be counterproductive, since it would mitigate the negative effects of pathogens but at the same time it would reduce sperm motility. Altogether the concept of shared microbiomes, the so called seminovaginal microbiome, is a new concept which requires further investigation since few and very heterogenous studies have been performed simultaneously in both partners and the current knowledge is inconclusive and warrants future studies to unravel microbial's function in modulating couple's microenvironment.

With the systematic search of microbiome studies performed in a low microbial biomass site in the human reproductive tract, as human endometrium as a study model, we provide an initial framework to establish the much-needed guidelines and best practices for designing and conducting low biomass microbiome studies and to draw attention to the weak points that could be improved or avoided to ensure that quality microbiome research in reproductive field is undertaken (**Study IV**). In general, the current studies are barely comparable and do not include detailed documentation of the study population, sample type and collection, data processing, and analysis workflow. Also, appropriate positive and negative controls are vital and mandatory to fully detect contaminants and to distinguish the low-abundance microorganisms that truly originate from the sampling site. Thus, we should aim transparency in presenting study results, providing raw data together with metadata.

General limitations

There are some limitations that should be considered:

- We analysed microbial composition of testicular sperm samples from men with infertility, whose testis microbiome could be altered (**Study I**). Thus, knowledge of the healthy commensal microbiome in the human testicles is lacking.
- Mid-stream urination and masturbation involve the urethra, which harbours the urethral microbiome and might influence the detection of pure seminal microbiome (**Study II**). To overcome this limitation, we also included urine samples into the analysis. Catheterisation and seminal vesicle aspiration would be more suitable collection methods to elucidate seminal microbial origin; however, not feasible in a daily clinical setting.
- Obtaining sufficient DNA yield from semen samples presents a challenge during microbiome sequencing. This difficulty in obtaining adequate amount of bacterial DNA complicates the sequencing process and requires larger sample material and more participants to compensate for potential sample dropouts (**Study II**).
- Systematic search of seminovaginal microbiome did not result in conclusive results due to small sample size studies, big heterogeneity between studies, and lack of proper positive and negative controls to account for contamination, making the findings of the seminovaginal microbiome in health and disease inconclusive (**Study III**).
- Marker gene amplification and sequencing provides a low-resolution information of microbial communities and limited functional analyses with no information about bacterial viability (**Study IV**). The use of metagenome sequencing would overcome this limitation, although the analysis complexity and costs would rise significantly.

Future directions

There are some points to consider for future research in male reproductive microbiome:

- Understanding what constitutes a healthy seminal microbiome is a key starting point. The focus should be on defining a "core" microbiome in a large cohort of healthy fertile men.
- New studies analysing the whole metagenome would identify, in addition to bacteria, also viruses, archaea, fungi, and other small eukaryotes to provide the full profile of seminal microbiome.
- There is a need to carry out longitudinal studies that could provide insight into how the male reproductive microbiome evolves over time, its stability, and its impact on health and disease.
- Information of lifestyle factors like diet, stress, exercise, substance use, and sexual practices should be considered in microbiome studies as they could potentially impact microbial composition. Understanding these influences will elucidate modifiable risk factors.
- Mechanistic studies are needed to unravel how shifts in the microbial composition impact sperm quality and male fertility, possibly by analysing the expressed genes within a microbial community (i.e., metatranscriptomics) and characterising the metabolites generated by microbes (i.e., meta-metabolome). Also, further research is needed to understand the viability of the microbes detected by NGS, as detection of microbial DNA does not necessarily equate to the presence of a live microorganism.
- Emerging evidence suggests that the gut microbiome can influence distant organs, including the testes, through the gut-testes axis, affecting male fertility.

Unravelling the role of the gut microbiome in the maintenance of normal testicular function would be valuable.

- Once the nature of the seminal microbiome is characterised, studies can be initiated to manipulate the microbial communities to improve male health and fertility. RCTs are required to establish the efficacy and safety of probiotic use in male health management.

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CONCLUSIONS

This Doctoral Thesis provides a comprehensive overview of the diversity and origin of the microbiome in the male genitourinary system via high-throughput omics analyses. Altogether these findings provide a step closer in describing and understanding the microbial landscape in human semen.

Specific conclusions

- The male reproductive tract (i.e., semen and testicles) harbours a polymicrobial communities with a low biomass but considerable diversity and abundant contamination.
- The genitourinary system contributes to the composition of the seminal microbiome as revealed by the bacterial genera shared with the identified sperm-specific microbiome, and between paired semen and urine samples before and after vasectomy. Further, sterilisation procedure of vasectomy could impact male health via modulating the seminal microenvironment.
- Our systematic review highlights that sexual exchange of microbes within couples influences the microbiome of both male and female reproductive tracts, indicating a complex interplay that might impact reproductive health and its outcomes. Nevertheless, the results are preliminary due to the heterogeneity, low sample size, and relatively few studies in the field. Further, seminal and vaginal microbiomes have traditionally been studied separately, thus future research should approach them as a holistic system, where both sites are complementing the microbial composition.

- The reproductive microbiome field requires clear, reproducible, and well-controlled study design to elucidate the healthy seminal microbiome and discern microbial tourists, invaders, or residents within the male reproductive system.